Diagnosis of pulmonary injury and infection by exhaled breath analysis
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Diagnosis of Pulmonary Injury and Infection by Exhaled Breath Analysis

Lieuwe D.J. Bos
DIAGNOSIS OF PULMONARY INJURY AND INFECTION
BY EXHALED BREATH ANALYSIS

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit van Amsterdam
op gezag van de Rector Magnificus
prof. dr. D.C. van den Boom
ten overstaan van een door het college voor promoties ingestelde
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Faculteit der Geneeskunde
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"I suppose that if all you have is a hammer everything looks like a nail"

Abraham Maslow
The Psychology of Science
1966
Part I

Introduction
Chapter 1.

General introduction and outline of the thesis

Adapted from:
Yearbook of Intensive Care and Emergency Medicine 2012

Lieuwe DJ Bos
Abstract

Signs and symptoms of acute respiratory distress syndrome or pneumonia are preceded by local activation of various molecular pathways, which could provide diagnostic biological markers. This includes volatile metabolites that can be separated, identified and quantified by gas–chromatography and mass–spectrometry (GC–MS). Exhaled breath condensate can be used to investigate both volatile and non-volatile organic compounds. These techniques have recently been used to separate patients with acute lung injury from patients with uninjured lungs, to predict pulmonary infection, and even to discriminate between different respiratory pathogens. In this review, we pinpoint the aims, pros and cons, and perspective of currently available technologies on exhaled metabolic profiling in critically ill patients.

Although the first clinical studies on the diagnostic accuracy of exhaled breath analysis showed good results, methodological and biological validation is needed. First, methodological recommendations were not always followed strictly which might result in false–discoveries. Second, since metabolism is the end–product of gene–expression, protein function and physiological landscape, confounding factors may have biased patient studies so far. A more translational approach in breathomics research in mechanically ventilated patients is advisable, combining clinical trials with preclinical in–vivo and in–vitro experiments as well as combining metabolite discovery by GC–MS with disease diagnosis by pattern recognition with electronic noses. Clinical trials might use a combination of different technologies, including GC–MS and tailor made bedside analytical tools, in order to provide probabilistic evidence (positive– or negative predictive values) for clinical decision–making.
Introduction

Critically ill patients frequently develop acute lung injury (ALI) [1] or ventilator–associated pneumonia (VAP) [2]. The diagnostic assessment of these conditions is complex, leading to suboptimal clinical management. ALI is diagnosed according to the American–European consensus criteria, which include acute non–cardiogenic bilateral pulmonary infiltrates on chest radiography and hypoxia [3, 4]. VAP is suspected when sputum production increases or changes, in combination with new or changed pulmonary infiltrates on chest radiography and hypoxia [2]. Unfortunately, these clinical signs and symptoms are all far from specific and occur in a relatively late stage of disease, hampering accurate and early diagnosis [5, 6].

It is likely that signs and symptoms of ALI and VAP are preceded by local activation of various molecular pathways [7-11]. Monitoring of these so–called biological markers has the potential to improve the diagnostic process in many ways. Ideally a biological marker is sensitive to early pathophysiologic changes and specific for disease. Assessment of biological markers should preferably be rapid as well as non–invasive and cheap to allow for frequent monitoring [12]. While several biological markers were suggested to have the potential to assist in diagnosing ALI and VAP, unfortunately, most of them have been shown to have an unacceptable low diagnostic accuracy [13-16].

In the Middle Ages, physicians depended heavily on their senses. Color, taste, and smell were their biological markers [17]. Although outdated, their sensing provided a quick, non–invasive and integrative view on biochemical processes without additional costs, processing and analysis. The emerging possibilities of “smelling” hundreds to thousands of biological markers in exhaled air to diagnose pulmonary diseases in intubated and mechanically ventilated critically ill patients are spectacular, but research is facing huge challenges. In this review, we pinpoint the aims, pros and cons, and perspective of currently available technologies on exhaled metabolic profiling in critically ill patients. A translational roadmap for further biomedical and technological research is presented.
Use of biological markers

The medical usage of biological markers critically depends on the clinical objectives [18]. Pulmonary inflammation due to ARDS or VAP should not be missed, thus a rapid bedside test should at first be very sensitive. A relatively low specificity could still allow for clinical application because a high negative predictive value with an acceptable positive predictive value can exclude patients from treatment while adequately treat true-positive patients. The limitations of a low specificity are prescription of antibiotics in false-positive pneumonia, leading to increased bacterial resistance, and the usage of very low tidal volumes in false-positive ARDS, resulting in high carbon dioxide levels. A test with similar test-characteristics as the gold standard could add to the available clinical diagnostics if this test is more rapid, continuously available, less invasive or cheaper.

A second objective would be better phenotyping of patients with ARDS or VAP. In pneumonia, a very specific test (very high positive-predictive value) for a particular strain of bacteria is of added value because antibiotic therapy can be focused from broad- to narrow-spectrum. In ARDS, a specific test for a subtype of pulmonary inflammation might allow for phenotype-targeted therapy. In other words, if the test provides evidence for the involvement of a metabolic that is important in the pathogenesis of ARDS in some patients, this test could provide a target for intervention.

A third aim of biological markers is the monitoring of treatment response and adjustment of therapy. Antibiotic treatment might be stopped in pneumonia when biological markers return to normal, hereby limiting antibiotic usage. Furthermore, a different treatment strategy might be initiated rapidly after the primary therapeutic strategy failed to show a biological response.

Biological markers in blood

Numerous studies have tested the diagnostic accuracy of biological markers in blood for pulmonary complications in critically ill patients. Except for procalcitonin, which was found to have some value in the diagnosis and treatment response of VAP [19, 20], other candidate biological markers in blood were found to have an unacceptably low diagnostic accuracy [13-15]. This may not come as a surprise because blood levels of biological
markers may only partly, if at all, reflect pulmonary changes. Pulmonary inflammatory responses with ARDS and VAP are highly compartmentalized [7, 10, 11]. Indeed, while strong and early pro-inflammatory reactions are seen in broncho-alveolar lavage fluid (BALF) with ARDS and VAP, systemic levels of inflammatory mediators do hardly change before clinical manifestation.

Second, one or two systemic biological markers may not sufficiently capture the complexity of ARDS or VAP. The pathophysiology of ARDS and VAP compromises numerous biological processes, including but not restricted to inflammation, oxidative stress, coagulation and apoptosis [21, 22]. Traditional studies on single proteins or pathways therefore may have very limited potential because the complexity of pulmonary disease cannot be captured. In the light of the dynamics and adaptive capacities of all biological systems, supposed molecular or cellular “alterations” should probably always be seen in the context of state of the system as a whole [23].

Third, a single measurement of a biological marker in blood heavily disregards the rapid dynamics of (development of) pulmonary diseases [24]. While use of multiple biological markers may improve diagnostics the rapid dynamics of critical illness remain ignored. Also, while blood is relatively easily available, frequent blood sampling and analysis in patients with or at risk for ARDS or VAP are relatively expensive and time-consuming.

**Biological markers in lungs**

There is good evidence that the lungs, rather than blood, should be assessed when aiming for early and accurate diagnosis of ARDS or VAP. BALF can be obtained in intubated and mechanically ventilated patients using directed or non-directed techniques [25]. Directed BALF has the advantage of sampling biological fluids in specific compartments of the lung but requires specialized personnel, investment of time and is not without risk for the critically ill patient. Non-directed lung lavage is relatively fast and easy to perform but may be still too invasive for frequent assessment.

Different biological pathways should be investigated simultaneously to acquire a broad biological perspective [26]. Recently, proteins in BALF have
been profiled collectively using new analytical techniques (proteomics), allowing for biological marker discovery and better understanding of the host-response [27].

“Omics” and systems biology

“Omics”–studies represent the integrated assessment of the biochemistry within a domain of complex organisms (genomics, transcriptomics, proteomics and metabolomics). These techniques are purposely not hypothesis–driven (i.e., they are unbiased) and can be used to discover biological markers of pathophysiological pathways [28]. Systems biology focuses on combinations of different “omics”–domains seeking a deeper understanding in complex biological systems providing a top–down view of biochemical processes combined with mathematical and computational methods for modeling of structures and processes. Integrating these with clinical parameters by a multiscale analysis is now called ‘systems medicine’ [29].

Metabolomics was recently described as “the global assessment of endogenous metabolites within a biologic system and represents a “snapshot” reading of gene function, enzyme activity and physiological landscape” [30]. The metabolome is very sensitive to physiological and pathophysiological changes because it is an end–product of the genome, transcriptome and proteome combined. This could possibly limit the specificity of the metabolome. Nuclear magnetic resonance (NMR) spectroscopy and liquid–chromatography/mass-spectrometry (LC–MS) can be used to identify hundreds of metabolites in any biological material. As with any “omics” technique, it remains a challenge to avoid false discoveries when applying metabolomics. Recommendations concerning bias, sample size, multiple testing and model fitting should be followed strictly [31, 32]. Since metabolism is an ancient and highly conserved biological mechanism, results can often be translated between mammalian species [33].

Up till now, some research has focused on metabolomics in critically ill patients. A NMR–based metabolomic method in plasma was described to aid detection of the systemic inflammatory response syndrome (SIRS) versus multi–organ failure (MOF) based on abnormal metabolic
signatures [34]. It was possible to discriminate SIRS and MOF patients, suggesting that an NMR–based metabolomic approach can be developed to diagnose the disease progress of critically ill patients. In a rat-cecal ligation and puncture model metabolomics showed a 100% sensitivity and specificity when compared to sham-operated animals [35]. Similar results were found in a mouse model of inflammatory lung injury [36]. These results were further supported by a clinical study showing that NMR in plasma could generate quantitative data sets that revealed differences between patients with ARDS and healthy subjects on the level of several metabolites [37]. Importantly, some metabolites were associated with acute physiology scores and ventilator–free days. This study clearly demonstrates the feasibility of plasma NMR quantitative metabolomics since it yields a physiologically relevant metabolite data set that distinguished disease from health. Of note, several metabolites are volatile and thereby eliminated via the lung.

**Breath metabolomics**

Breath contains thousands of volatile organic compounds (VOCs), metabolites in gas–phase produced by both physiological and pathophysiological processes [38, 39]. VOC–patterns identified by smell have been used to diagnose disease and intoxication for ages (e.g. scent of acetone in diabetes mellitus) [40]. Alteration of exhaled VOCs can be the consequence of changed systemic metabolism (e.g., diabetes mellitus) or due to pulmonary metabolomic shift [39]. Micro–organisms (e.g., bacteria) present in the airways also produce volatile molecules, which may be species specific [41, 42]. Thus the exhaled breath contains the composite signal of host–metabolism, as part of the host–response, and bacterial metabolism, which may interact [42]. It should be noted that due to the water and fat-solubility of VOCs the breath concentration is not a direct representation of the tissue concentration per se. A variety of techniques has been used to link disease to changed VOC composition of the exhaled air, including gas–chromatography and mass–spectrometry (GC–MS), Ion–molecule reaction mass–spectrometry (IMR–MS), and electronic nose techniques [43]. Both volatile and non–volatile metabolites can be studied in exhaled breath condensate (EBC) [44]. These techniques, including advantages and disadvantages, will be described on the following pages and are summarized in table 1. Head to head comparison of the diagnostic
value of the different techniques cannot be performed because of the low number of studies, differences in research aims and methodological inconsistencies.

Gas–chromatography and mass–spectrometry

GC–MS is considered the gold standard for the detection, separation and identification of large volatile organic compounds. With gas-chromatography molecules are carried with an inert gas (e.g., helium) through a column: molecules are separated by volatility and interaction with the stationary phase of the column. During mass-spectrometry components are fragmented into charged particles, carried through an electromagnetic field and quantitatively detected [45].

The lower limit of detection can be improved using pre-concentration. However, storage bears the risk of decomposition and/or loss of compounds. Considering the rapid dynamics of critically ill patients, test results should be available within minutes after measurement. This is not yet feasible, and storage and transport of air samples is a challenge. These factors still limit the clinical applicability of GC–MS in monitoring critically ill patients. Nevertheless, GC–MS remains essential for VOC identification and therefore for the understanding of pathophysiological pathways.

GC–MS has been used to study volatile metabolites in intubated and mechanically ventilated patients (table 2 [46, 47]). A decrease in isoprene production has been found in ARDS patients and an increased n–pentane/isoprene ratio during development of VAP [46]. Increased isoprene concentration is considered a marker of activation of neutrophils [48]. It was suggested that impaired cholesterol synthesis late in the course of ARDS could explain for the surprisingly low levels of isoprene. N–pentane is an end product of lipid peroxidation and could reflect an increase in oxidative stress. Although isoprene, pentane and acetone are about the most abundant VOCs in the exhaled breath, focusing on just these compounds disregards the potential benefit of composite information provided by “omics” strategies. All detected molecules should be presented to data reduction and classification algorithms in order to capture the complexity of the biological material and to obtain maximal diagnostic accuracy while limiting bias [31].
In short, GC–MS can be used to separate, identify and quantify volatile organic compounds in the exhaled breath. Clinical applicability is limited because it is expensive, slow, labor–intensive and requires specialized personnel. GC–MS will mostly be used to identify molecular targets in the exhaled breath.

**Ion–molecule reaction mass–spectrometry and other rapid detection methods**

Ion–molecule reaction mass–spectrometry (IMR–MS) has been proposed as a fast and sensitive alternative analytical method for the detection of molecules in gas–phase [49]. With IMR–MS volatile molecules are ionized with a precursor ion and moved towards a detector (mass-spectrometer). Volatile molecules are thus detected based on reactivity with the precursor ion and mass [49]. IMR–MS can be divided into different technologies based on the type of precursor ion: H3O⁺ called proton–transfer reaction mass–spectrometry (PTR–MS), H3O⁺/NO⁺/O2⁺ called selected ion flow tube mass–spectrometry (SIFT–MS) and others using krypton mercury or xenon [50].

The fast reaction time (20 ms), on–site application and continuous registration bring IMR–MS closer to clinical applicability. However, at present IMR–MS is not yet an alternative for GC–MS analyses because IMR–MS does only detect gases which react with the precursor ion. Furthermore, only mass-to-charge ratios are insufficient for identification of specific VOCs. Roughly the same advantages and limitations apply to ion–mobility spectroscopy (I–MS). In this technology, the ionized gas is let into a tube at specific intervals, in which the ions collide with drift gas molecules travelling in the opposite direction. Based on size and shape, ions are decelerated resulting in different ion drift times [51]. A summary of the advantages and disadvantages of the different technologies can be found in table 1.

The potential of rapid mass–spectrometry based technologies for breath analysis in mechanically ventilated patients was illustrated through the continuous analyses of air from the ventilatory circuit for up to 120 minutes [50]. In this study, several VOCs were identified and quantified, but no patient groups were compared.
Another method to analyze these datasets is to use pattern-recognition software. This is based on the concept that diagnostic assessment does not require identification of individual molecular components, rather than being dependent on accurate pattern recognition [52]. All peaks and intensities are combined into one algorithm, which is subsequently used for diagnostic purposes. This approach was recently used in rats challenged with lipopolysaccharide [53]. In this study, exhaled breath profiling with I–MS discriminated extremely well between rats with inflammatory response syndrome from healthy controls.

SIFT–MS has previously been used to discriminate between different bacterial species in vitro. This could provide a rapid alternative to traditional, culture dependent, pathogen detection. Several papers showed differences in around twenty volatile organic compounds between several cultured bacterial species [54-56]. A single VOC did not sufficiently separate bacterial species, however a pattern-recognition based approach resulted in very good discrimination between potential pathogens [57]. The biological materials used in these studies remain confined to spiked blood samples and the results should be validated using prospectively collected patient material.

Shortly, IMR–MS and I–MS are rapid tests that can be used to identify some volatile organic compounds in the exhaled breath. Because IMR–MS and I–MS technique can be miniaturized and the analysis is continuous, it could be used as a diagnostic test. However, compared to GC–MS these techniques are more selective and unknown compounds cannot be identified with certainty, hereby limiting it’s applicability for pathophysiological research.
<table>
<thead>
<tr>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GC–MS</strong></td>
<td>1. Slow and labor intensive</td>
</tr>
<tr>
<td>1. Identification of new compounds, thus allows for biomarker discovery</td>
<td>2. Not available at bedside</td>
</tr>
<tr>
<td>2. High sensitivity</td>
<td>3. Need for pre-concentration and transport</td>
</tr>
<tr>
<td>3. Wide range of application</td>
<td></td>
</tr>
<tr>
<td><strong>IMR–MS</strong></td>
<td>1. Selectivity for reaction between VOC and precursor ion</td>
</tr>
<tr>
<td>1. Identification of known compounds</td>
<td>2. Limited identification</td>
</tr>
<tr>
<td>2. Rapid measurement</td>
<td></td>
</tr>
<tr>
<td>3. Continuous analysis</td>
<td></td>
</tr>
<tr>
<td><strong>PTR–MS</strong></td>
<td>1. Selectivity for reaction between VOC and proton</td>
</tr>
<tr>
<td>1. Identification of known compounds</td>
<td>2. Limited identification</td>
</tr>
<tr>
<td>2. Rapid measurement</td>
<td></td>
</tr>
<tr>
<td>3. Continuous analysis</td>
<td></td>
</tr>
<tr>
<td><strong>SIFT–MS</strong></td>
<td>1. Selectivity for reaction between VOC and precursor ion</td>
</tr>
<tr>
<td>1. Identification of known compounds</td>
<td>2. Limited identification</td>
</tr>
<tr>
<td>2. Rapid measurement</td>
<td></td>
</tr>
<tr>
<td>3. Continuous analysis</td>
<td></td>
</tr>
<tr>
<td><strong>I–MS</strong></td>
<td>1. High selectivity towards very volatile molecules</td>
</tr>
<tr>
<td>1. Identification of known compounds</td>
<td>2. Limited identification</td>
</tr>
<tr>
<td>2. Rapid measurement</td>
<td></td>
</tr>
<tr>
<td>3. Continuous analysis</td>
<td></td>
</tr>
<tr>
<td><strong>eNose</strong></td>
<td>1. No identification of compounds</td>
</tr>
<tr>
<td>1. Rapid measurement</td>
<td>2. Lower sensitivity with current sensors</td>
</tr>
<tr>
<td>2. Portable and easy to use</td>
<td></td>
</tr>
<tr>
<td>3. New sensors development</td>
<td></td>
</tr>
<tr>
<td>4. Continuous analysis</td>
<td></td>
</tr>
<tr>
<td><strong>EBC</strong></td>
<td>1. Collection and analysis are slow</td>
</tr>
<tr>
<td>1. Liquid sample is obtained so traditional analyses can be performed</td>
<td>2. Continuous analysis is impossible</td>
</tr>
<tr>
<td>2. Combination with NMR allows for profiling</td>
<td></td>
</tr>
</tbody>
</table>
Table 2: Volatile organic compounds detected in exhaled air of mechanically ventilated patients, as described in literature [34] [50] [47]

<table>
<thead>
<tr>
<th>Compound</th>
<th>Suspected origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone</td>
<td>Enhanced metabolism</td>
</tr>
<tr>
<td>2,3-Dimethylbutane</td>
<td>Unknown</td>
</tr>
<tr>
<td>2,4-Dimethylpentane</td>
<td>Unknown</td>
</tr>
<tr>
<td>2-Butanone</td>
<td>Unknown</td>
</tr>
<tr>
<td>2-Methylbutane</td>
<td>Respiratory delivery system</td>
</tr>
<tr>
<td>2-Propenal</td>
<td>Unknown</td>
</tr>
<tr>
<td>Acetaldehyde</td>
<td>Unknown</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>Smoking</td>
</tr>
<tr>
<td>Butanal</td>
<td>Unknown</td>
</tr>
<tr>
<td>Butane</td>
<td>Oxidative stress</td>
</tr>
<tr>
<td>Cyclohexane</td>
<td>Smoking</td>
</tr>
<tr>
<td>Cyclohexanone</td>
<td>Respiratory delivery system</td>
</tr>
<tr>
<td>Ethanol</td>
<td>Endogeneous or hospital air</td>
</tr>
<tr>
<td>Heptane</td>
<td>Oxidative stress</td>
</tr>
<tr>
<td>Hexanal</td>
<td>Unknown</td>
</tr>
<tr>
<td>Hexane</td>
<td>Unknown</td>
</tr>
<tr>
<td>Isoflurane</td>
<td>Anesthesia</td>
</tr>
<tr>
<td>Isoprene</td>
<td>Cholesterol metabolism</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>Respiratory delivery system</td>
</tr>
<tr>
<td>Methanol</td>
<td>Unknown</td>
</tr>
<tr>
<td>N-hexane</td>
<td>Respiratory delivery system</td>
</tr>
<tr>
<td>Pentanal</td>
<td>Unknown</td>
</tr>
<tr>
<td>Pentane</td>
<td>Oxidative stress</td>
</tr>
<tr>
<td>Propanal</td>
<td>Unknown</td>
</tr>
<tr>
<td>Propane</td>
<td>Unknown</td>
</tr>
<tr>
<td>Toluene</td>
<td>Smoking</td>
</tr>
</tbody>
</table>
**Electronic nose**

Electronic noses (eNose), named after their similarities with mammalian olfactory system, integratively capture complex VOC mixtures using an array of different sensors [43]. Humans, not even the most versatile smellers known to nature, are able to discriminate 1 trillion olfactory stimuli with just 400 receptors [58]. Chemical sensors have individual sensitivities and specificities for multiple VOCs. The composite signal of all sensors can be analyzed using pattern-recognition algorithms. eNose analysis of breath results in a unique fingerprint of exhaled metabolites, called a breath-print. Subsequently, these breath-prints can be used for diagnostic and monitoring purposes, which do not require identification of individual molecular constituents.

Metal oxides, conducting polymers, optical and infra-red spectroscopy have been used as sensors. Electronic noses can be miniaturized and might allow for continuous analyses. Data are available in real-time and electronic noses are relatively easy to use. Indeed, eNoses are very attractive from a clinician point of view [39]. Identification and quantification of specific compounds is not necessary for diagnosis and monitoring as long as patterns are diagnostic for particular conditions. Although promising, several technical issues are to be considered regarding eNoses, including the fact that sensors in use at present have a limited sensitivity and specificity for VOCs, are not interchangeable between devices and could “drift” over time. Sampling techniques should be adapted to the clinical setting and the disease of interest.

Several volatile compounds have been linked to metabolic activity of relevant bacterial species including *Pseudomonas aeruginosa* and *Staphylococcus aureus* [39]. Assessment of a large quantity of VOCs combined with pattern recognition software leads to good discrimination between bacterial species [59]. Different species of bacteria can be discriminated *in vitro* based on integrative analysis of volatile metabolites using an electronic nose [60-62]. Interestingly, distinct metabolic alterations have been reported for methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecalis* as compared to antibiotic susceptible bacteria of the same species [63].
Patterns of VOCs in exhaled breath of intubated and mechanically ventilated patients undergoing surgery are associated with the clinical pneumonia infection score, a sensitive marker for VAP [64]. Real-time pathogen detection has been reported in mechanically ventilated critically ill patients with pneumonia, showing good *in vivo* discrimination between *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Acinobacter baumanii* and *Acinobacter lwoffi* [65] (figure 1). Air was acquired through a suction catheter for the purpose of this study, compromising the non-invasiveness of breath sampling and the possibility of continuous analysis. Although GC–MS analysis of exhaled breath was not performed (i.e., the specific compounds remained unknown), these studies illustrate the diagnostic potential of volatile metabolites generated by airway pathogens and/or host response.

**Figure 1: Discrimination between pathogens *in vivo***

During a preliminary proof of concept study we recently showed the potential of eNoses to discriminate ARDS patients from critically ill patients without lungs injury (figure 2) [66]. These results are very encouraging since the patient groups were small and highly heterogeneous, but validation is very much needed. In this study, air was collected for one minute via a disposable T-piece connector placed between the endotracheal tube and the heat-moist exchanger. This methodology allowed for fully non-invasive and continuous sampling, suggesting electronic nose technology may already be very close to clinical applicability.

**Figure 2:** Discrimination between ARDS and controls

*Discrimination between acute lung injury patients and controls by electronic nose analysis. The X- and Y-axis represent principal components obtained by data reduction. Each point represents a critically ill patient: □, controls; Δ, acute lung injury patients. Each group is connected to a centroid.*

eNoses can provide rapid analysis of complex volatile mixtures as the breath. The devices are portable, easy to use and sensors can be tailor-made. Furthermore, in intubated and mechanically ventilated patients,
continuous exhaled breath analysis is possible. Therefore, electronic noses are potentially suitable as diagnostic and/or monitoring instruments. However, electronic noses cannot identify volatile organic compounds making them less important for understanding the molecular mechanism altered in disease.

**Exhaled breath condensate**

Exhaled breath condensate originates from lining fluid in the upper or lower airways [44]. EBC therefore contains a large number of peptides and metabolic products. Concentrations of diverse peptides and other molecules (e.g., interleukines, isoprostanes and cytokeratins) in EBC have found to be altered in acute lung injury [67-69]. One study showed leukotriene B4 in EBC to be increased in children with community-acquired pneumonia [70].

The association between pneumonia, ALI and metabolite concentrations in EBC has not yet been investigated. In respiratory research, metabolomic profiling of EBC fluid by NMR–spectroscopy showed excellent results in distinguishing asthma, COPD, and cystic fibrosis patients from controls. In a similar way stable from unstable cystic fibrosis patients could be separated [71-73]. NMR–spectroscopy records the interaction of radiofrequency electromagnetic radiation within the nuclei of atoms in a strong magnetic field. The nuclei within a biomolecule can hereby be detected and used to determine the molecular structure, conformation and dynamics. Identification might be possible, as with GC–MS, by comparing spectra with a reference library.

A major advantage of EBC analysis is that the more traditional, well-understood biomarkers can be measured non-invasively. It is therefore a diagnostic tool with great potential for pulmonary disease, also in critically ill patients. However, collection of EBC remains challenging in mechanically ventilated patients. For instance, air should not be artificially humidified to obtain a meaningful signal. Therefore, humidifying–systems should not be used, which may be unwanted One other disadvantage of EBC collection is that collection systems are rather large. In addition, continuous analysis of complex mixtures does not seem possible.
Perspective

Volatile organic compounds in the exhaled breath can be separated, identified and quantified by gas–chromatography and mass–spectrometry. This remains the gold standard for pathophysiological research. Several other technologies can be considered as diagnostic tools because they are rapid and easier to handle: IMR–MS and I–MS can be used for pattern recognition as well as VOC detection while electronic noses rely on pattern recognition only. Non-volatile organic compounds can be investigated using exhaled breath condensate.

Momentarily, there are two large gaps in the exhaled breath research: firstly there is little consistency in research aims and methodology between basic research and clinical trials, secondly biomedical researchers and the developers of new technologies are not in close contact. Future research on exhaled breath analysis in intubated and mechanically ventilated patients should approach volatile biomarker discovery using translational biology and translational technology (from bench to bedside and \textit{vice versa}) (figure 3).

VOCs can be discovered \textit{in vitro}, as demonstrated by head–space analysis of bacteria [41]. The head–space analysis of pulmonary fluids (e.g., BALF) also investigates (part of) the host response. These findings can be extended using “clean” \textit{in vivo} animal models (e.g., models of pneumonia or lung injury), in which two variables are added: the physiology of the respiratory system and the systemically produced volatile organic compounds. Clinical trials following STARD–guidelines are then necessary to validate the diagnostic accuracy of breath metabolomics in patients with co–morbidities and exposure to exogenous VOCs [74].

Translational technology should focus on matching technology to research aim. Fundamental research should use GC–MS for biomarker discovery and detailed study of (patho–) physiological processes. Clinical trials might use a combination of different technologies, including GC–MS and tailor made bedside analytical tools, in order to provide probabilistic evidence (positive– or negative predictive values) for clinical decision–making [32]. Tailor–made eNoses and/or diagnostic algorithms for SIFT-MS are to be produced when VOCs associated with disease have been identified and
validated [75].

No head-to-head comparison between the available analytical technologies for complex breath samples is available. The hereby proposed purposes for each technology imply that rapid analytical tools (for diagnosis and monitoring) should not be compared to GC–MS (pathophysiological research) but should be compared to each other. However, the problem with defining the gold-standard remains and might imply that we need more objective end-points.

**Figure 3:** Perspective

*Perspective for the development of breath analysis as a diagnostic test for pulmonary disease.*
Conclusions

Exhaled breath analysis has potential as a diagnostic and monitoring tool in intubated and mechanically ventilated critically ill patients especially so because it is fully non-invasive and can be performed continuously. There is accumulating evidence for the diagnostic accuracy of breath analysis in several pulmonary diseases. Additionally, several VOCs have been linked with disease processes. However, there is still room for improvement in trial design and the use of the right technology for the research aims of the future studies. Special attention should be given to the coupling of biochemical pathways with the observed alterations in exhaled VOCs.
Outline of this thesis

The general aim of this thesis is to obtain more insight into changes of volatile organic compound composition during ARDS and pulmonary infection.

- In Chapter 2, we give an introduction in the analysis of exhaled breath in ventilated ICU-patients. Special consideration is given to the previously studied volatile compounds and the techniques of measurement that are used for the experiments that are described in this thesis.
- In Chapter 3, we describe a new, simplified method for exhaled breath collection in ventilated patients. This method was used in all subsequent chapters.
- In Chapter 4, in an attempt to discriminate between patients with and without ARDS, we integratively measure the VOCs in exhaled breath using a commercially available electronic nose.
- In Chapter 5, we investigate the alterations in exhaled volatile organic compounds during the development of systemic and local inflammation. For this we use a rat model with intravenous or intratracheal lipopolysaccharide injection and mechanical ventilation.
- In Chapter 6, we identify VOCs that may be used for the diagnosis of ARDS. We aim to find a small panel of biomarkers and externally validated the accuracy of these volatile metabolites.
- In Chapter 7, we review the literature for potential volatile biomarkers for bacterial presence.
- In Chapter 8, we describe the in-vitro examination of volatile organic compounds analyzed by electronic nose for the diagnosis of ventilator-associated pneumonia.
- In Chapter 9, we measure VOCs in the exhaled breath of ventilated ICU-patients and analyze their association with pneumonia.
- In Chapter 10 and 11, we summarize and discuss the results from the previous chapters.
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performance and potential of ion mobility spectrometry. J Breath Res 3: 036004


Chapter 2.

Development of breath analysis in intubated and ventilated intensive care unit patients

Not published

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Abstract

Exhaled air contains a complex mixture of molecules that may change with disease. Breath analysis has shifted from detection of abundantly present molecules like carbon dioxide to monitoring of scanty volatile organic compounds, and from a focus on single molecules to a broader look at mixtures of biological markers. Breath analysis is now used to detect hundreds of exhaled metabolites, combinations of which have the potential to serve as markers of physiological and pathophysiological processes as they may reflect oxidative stress, inflammation, infection, and organ failure. While gas–chromatography and mass–spectrometry is used for discovery of volatile biological marker, this technique is impractical for daily practice since it is time–consuming and expensive. New techniques may, guided by gas–chromatography and mass–spectrometry discovery of volatile biomarkers, result in bedside, rapid diagnosis or monitoring of disease by a simple exhalation. Besides applications in the first line and on hospital wards, this may allow for continuous real-time monitoring in critically ill patients, especially when ventilated. This may result in a monitoring system for cellular and molecular processes like organ injury and infection that can momentarily only be extrapolated from alterations in macroscopic physiology.
Introduction

Most, if not all intensive care unit (ICU)–patients are under constant monitoring of physiological signs in an attempt to early detect deteriorations (mainly organ failure and infection) or responses to therapies. Unfortunately, presently used monitoring techniques are almost without exception limited to global parameters [1, 2]. Global parameters are the result of alterations in multiple molecular pathways and are therefore late and a–specific signs of dysfunction [2]. There is a growing need for assessment of molecular and/or cellular changes in order to interfere earlier and in a more homogenous group of patients [3, 4].

Assessment of molecular and cellular processes is difficult as it depends on off site biochemical analysis of blood or other bodily fluids. The fluid should be obtained and send to the lab for analysis. Sample collection, transport and analysis may cause delay in initiation or adaption of therapy; for example broncho–alveolar lavage cannot be performed at any given moment and it may take days before culture results are known. Delays are unwanted as therapy is most effective in an early phase of disease [5-8]. Ideally, monitoring should be continuous, rapid and online.

Physicians depended heavily on their senses before the era of technological innovation. Color, taste, and smell were their biological markers [9]. Although outdated, their sensing provided a quick, non–invasive and integrative view on biochemical processes without additional costs, processing and analysis. Several diseases are reported to induce a distinctive smell of breath (e.g. acetone during keto–acidosis and fetor hepaticus) [10]. Exhaled breath contains hundreds sorts of molecules in gas phase. The composition of this mixture can be altered in disease. Nowadays, the development of biochemical sensors and enhanced data–analysis allows for artificial smelling of exhaled breath [11]. Diagnosis and monitoring based on exhaled breath analysis may be attractive for the intensive care physician as it is non–invasive [12, 13].

In this chapter we discuss the emerging field of exhaled breath analysis for monitoring of patients on the ICU. Exhaled molecules of interest will be discussed from high to low abundance (figure 1); from known into unknown territory. Every molecule will teach a principle of exhaled breath
analysis that can be applied to novel techniques. We aim to provide an overview of the current status and future trends.

**Figure 1: Composition of exhaled breath**

![Diagram showing the composition of exhaled breath with labels for CO2, Cytokines, Metabolites, Water, CO, NO, VOCs, and Breathomics.]

**Carbon dioxide**

Carbon dioxide (CO2) is the only largely endogenous gas that is routinely measured in the breath of intubated and ventilated patients. Indeed, continuous capnometry has an important place in respiratory monitoring [1]. CO2 is the result of aerobic metabolism and transported to the lung by venous return. Therefore, the main applications of capnography are (1) monitoring airway patency and ventilation, (2) assessment of alveolar dead space and (3) during cardiopulmonary resuscitation [14]. The applications correspond to the three principles that are at the foundation of capnography: (1) the lung is the only compartment in which CO2 normally and continuously exits the circulation; (2) delivery depends on the amount and homogeneity of pulmonary blood flow and (3) without aerobic metabolism CO2 production ceases. The success story of capnography teaches us the potential of continuous monitoring.


**Carbon monoxide**

Carbon monoxide (CO) is known as an exogenous intoxicative compound. The fact that CO is also produced endogenously by heme oxygenase in the process of heme degradation is less well known [15]. This process occurs mainly in the liver. However, heme oxygenase is also present in vascular endothelium and alveolar macrophages of the lungs and can be up-regulated by oxidative stress and inflammation [15]. CO is exhaled and may be a breath marker of inflammation. Indeed, CO was found in higher concentrations in respiratory infections, asthma and exacerbations of cystic fibrosis (CF) [15-18]. Smoking is a major confounder as it independently leads to very high CO concentrations in exhaled breath with a half-life of around 6 hours [15]. Interestingly, CO was found to negatively modulate inflammation in hyperoxic lung injury [19]. CO is thus associated with inflammation but is a mediator in the anti-inflammatory response [20]. CO teaches us that exhaled molecules can be of systemic or pulmonary origin and that volatile compounds may be biologically active.

**Nitric Oxide**

Exhaled nitric oxide (NO) is proposed as a diagnostic breath marker for the airway inflammation associated with asthma [21]. A major success of exhaled NO is that it predicts the clinical response to corticosteroid treatment in these patients [22]. NO is constantly produced by NO synthases (NOS) and acts as a modulator of arteriolar and bronchial tone, platelet aggregation and inflammation [23]. Endothelial cells have a relatively low output of NO while airway epithelial cells produce higher concentrations. The latter can be further induced through inflammation [24, 25]. Thiol-containing compounds may interact with NO and provide a temporal reservoir [26]. Importantly, the upper airways also contribute to the exhaled NO concentration.

An excellent recent review by Boshier *et al.* discussed the potential of NO as biological marker for acute lung injury [27]. One would expect a higher exhaled NO concentration as NOS activity increases with inflammation. However, predominantly lower exhaled NO levels are found in acute respiratory distress syndrome (ARDS) [27]. This may be explained by increased NO consumption by oxidative stress. NO reacts with superoxide
to produce peroxynitrate, leading to molecular damage, such as lipid peroxidation [28]. Besides, NO acts as an anti–oxidant by inhibition of lipid radical chain propagation [29]. Both lead to increased NO consumption and a lower netto exhalation. NO teaches us that exhaled molecules can be produced in different parts of the airways and that both higher and lower concentrations may be informative.

**Exhaled breath condensate**

Besides volatile water content, micron and sub–micron sized droplets emanate in exhaled breath [30]. It has been hypothesized that these droplets are blown from the epithelial lining fluid during turbulence or opening of collapsed airways [31]. Exhaled breath condensate (EBC) is a combination of droplets containing non–volatile molecules, diluted with condensed exhaled water content and substituted with water–soluble volatile organic compounds (VOCs) [31, 32]. Dilution, which can range from 20 to 30,000–fold, is a problem with EBC [33]. Three solutions are proposed: (1) standardize against a stable background molecule, such as urea [34], (2) use the ratio of multiple biomarkers (e.g. NO2–:NO3– or pH) and (3) use a qualitative test.

Several non–volatile biomarkers have been investigated in the EBC of mechanically ventilated ICU–patients. EBC levels of these markers should reflect concentrations in the epithelial lining fluid. Hydrogen peroxide (H2O2), isoprostane, NO2–, pH and lactate – markers of anaerobic metabolism and oxidative stress – are elevated in ARDS [35-38]. These markers may also be used to monitor treatment response [39, 40] and correlate well to values in broncho–alveolar lavage fluid (BALf) [41]. Inflammatory cytokines are also increased in the EBC of ARDS patients [42], but difficulties with the lower limit of detection and quantification complicate cytokine measurements in EBC [33].

Water–soluble volatile organic compounds can be found in high concentrations in EBC as these volatile molecules pass by during sample collection and dissolve into the water. High–throughput technology is nowadays available for the analysis of these small metabolites. Nuclear magnetic resonance (NMR) spectroscopy and liquid–chromatography and mass–spectrometry (LC–MS) can be used for liquid samples. For example,
NMR analysis of EBC showed good discrimination between patients with CF and controls and between stable and unstable CF [43]. One metabolite was not sufficient to discriminate. Rather, subtle changes in multiple metabolites reflected (unstable) disease. Since the underlying cellular and biochemical mechanisms of any disease are integrated into a complex network of interactions it is very probable that multiple biomarkers will outperform a single one. “Omics”–studies represent the integrated view of the biochemistry within a domain of complex organisms [44]. Metabolomics is the “global assessment of endogenous metabolites within a biologic system and represents a “snapshot” reading of gene function, enzyme activity and physiological landscape” [9]. EBC teaches us how to deal with the problem of dilution and the potential of an “omics” approach to discover novel molecular markers.

**Breath metabolomics**

It is not necessary to dissolve VOCs into water to perform metabolomics analysis. Volatile metabolites can be analysed directly in the exhaled breath, or absorbed onto a transport tube and analysed elsewhere. High–throughput analysis of exhaled breath is also called “breath metabolomics” or in short “breathomics”. Breath contains hundreds of VOCs, metabolites in gas–phase produced by both physiological and patho–physiological processes [45, 46]. Alteration of exhaled VOCs can be the consequence of changed systemic metabolism (see section on CO2) or due to pulmonary production (see section on CO and NO) [46]. Bacteria also produce volatile metabolites [47, 48]. Thus exhaled breath contains the composite signal of host–metabolism, as part of the host–response, and bacterial metabolism, which may interact [49]. Below we describe the two most important methodologies for VOC analysis that were used in this thesis; gas-chromatography and mass-spectrometry and electronic nose analysis.

**Gas–chromatograph and mass–spectrometry**

Gas–chromatography and mass–spectrometry (GC–MS) is considered the gold standard for the discovery of volatile organic compounds. In the following section, we will discuss the analytical principles and considerations in detail.
Trapping and pre-concentration

As the analytical machine is not available at the bedside, transport of the sample is required for the analysis of VOCs with GC-MS. VOCs can be contained in a glass syringe and injected into the gas-chromatograph, if done quickly [50]. VOCs cannot be stored over a longer period of time using this method as a syringe in not completely airtight and oxidation processes can occur, leading to loss and modification of the VOCs, respectively. Alternatively, VOCs can be trapped onto a sorbent material [51] such as Tenax (TA). However, all sorbent materials show selectivity towards certain types of VOCs. For example, Tenax is mainly hydrophobic and therefore polar compounds are not always trapped, although polar compounds are present if the air is humid, which suggest some trapping of water as well [51]. Volatiles with a very low boiling point show breakthrough (not trapped on the Tenax) if high concentrations are used [52]. Therefore, compounds that are polar and/or have a low boiling point cannot be determined quantitatively with trapping on Tenax. However, under the assumption that this bias is constant, semi-quantitative measurements can be performed. When VOCs are absorbed onto Tenax, they can be stored for at least two weeks at 4°C [53]. Volatile molecules can be released by thermal desorption of the tube filled with Tenax (figure 2). After re-focussing the breath sample onto a cold trap (with a lower mass, allowing for more rapid heatings and thus a shorter injection period) the sample can be injected into the gas-chromatograph through a heated liner.

Gas-chromatography

Gas-chromatography is used to separate the different VOCs within a breath sample so that they can be detected individually (see section on mass-spectrometry). For VOC analysis, typically a capillary column is used. In this long (i.e. 30m), thin (i.e..0.25 mm) column the molecules are carried by a gas (i.e. helium), which is called the “mobile phase”, towards the detector (figure 2). The time that a molecule travels through the column, called the retention time, is a representation of the chemical interaction of the molecule with the packing of the column, called the stationary phase. Many different types of stationary phases are available but for breath research a non-polar silicon polymer is mostly used (i.e.
Because chemical interaction alone results in insufficient separation and wide peaks (one type of molecule exits the column for a long period of time) temperature programs are used. This gives two advantages; better separation is obtained by a combination of chemical interaction with the stationary phase and boiling point; and the peaks can be narrowed as chemical interaction with the stationary phase rapidly become less likely as temperature increases.

**Mass-spectrometry**

There are different techniques for detection of VOCs that exit the column. For quantitative detection flame ionization detection gives the best results. Basically, the molecules that exit the column are burned and the ions that are generated are measured. However, for breath research the molecules of interest are not known and discovery is an important aspect of the analyses. Therefore, quadrupole mass-spectrometry can be used to tentatively identify the molecules that are detected, while maintaining (semi-) quantification. When a group of molecules, presumably of one molecular structure as they are separated by gas-chromatography, exit the column they are ionized by electron bombardment (electron ionization) (figure 2), although other forms of ionization are also available (e.g. chemical). Electron ionization results in a typical fragmentation pattern per molecule, which can be compared to a library of reference fragmentation patterns for known molecules (see identification). The ions are pushed in between the four parallel rods of the quadrupole mass-spectrometer that use an oscillating electrical field to selectively stabilize or destabilize the trajectories of certain masses of ions (figure 2). In other words, only ions with a certain mass-to-charge ratio (M/Z) pass through the quadrupole and can be detected electronically. Because the electrical field in the parallel rods oscillates very frequently a near-continuous measurement of all nominal masses within a certain range can be detected. Importantly, with adequate tuning of the machine, the semi-quantitative results of this mass-spectrometer are quite comparable over time.

**Processing of the GC-MS signal**

GC-MS analysis results in a three-dimensional matrix per sample (figure 2). The first dimension is the retention time and shows the separation of
the VOCs. The second dimension is the mass-to-charge ratio and displays the fragmentations of the VOCs. The third dimension is the intensity of the signal. After adjustment for background signals, it is key to reduce the number of dimensions to one to allow for statistical analysis. Ideally, one would like to create a list of concentrations per molecule per sample. The first step is to identify if a molecule is eluding from the GC by finding peaks in the different M/Z-windows of the MS. Under good chromatographical conditions, one compound exits the GC in such a way that it forms a Gaussian shaped pattern within the different M/Z-windows. That property can be used to detect peaks per M/Z by taking the first and second derivative; a well-separated peak passes zero in the first derivative and passes zero in close proximity on both sides in the second derivative.

The area under the peak, which is a semi-quantitative measure of the concentration, can be estimated by fitting a Gaussian function. This method eliminates one dimension; the retention time. The other dimension, the different M/Z windows, can be reduced quite simply by putting all peaks per M/Z in a wide format, resulting in a list of M/Z-peaks intensities, with a certain retention time. However, we cannot use these lists for statistical analyses because retention times can differ slightly between samples. Therefore, peaks first need to be matched across samples to allow for retention time correction. Portions of the chromatogram can be matched with the use of chemical standards and frequently present peaks that have a unique mass within a certain range of retention times. Information on the differences in retention times between the samples based on these matches can be used for retention time correction. Multiple iterations of linear and non-linear retention time corrections allow for optimal alignment of the samples and adequate grouping of the detected peaks. Additionally, it is plausible that ion-fragments meet the shape requirement in most samples but are not detected in a minority. With the information of the corrected retention time and the peaks that are detected in the other samples, the samples where the peak is “missing” can now be searched more sensitively for that ion-fragment. This limits the amount of missing data, which can be fatal for certain statistical classification methods (that for example require a normal distribution) and cross-validation methods (a peak classified cases in the training set, but is incorrectly missing in the cross-validation sample wherefore classification fails).
Figure 2: Gas-chromatography and mass-spectrometry

Breath analysis by means of GC-MS. From the upper left, clockwise: breath is collected and stored on a absorption tube that is desorbed to bring the molecules into the gas phase. The VOCs are separated by gas-chromatography and fragmented and detected by mass-spectrometry, which results in a three-dimensional matrix (mass over charge (M/Z), retention time and intensity (counts of ion-fragments)). By means of filtering and retention time correction, a one dimensional matrix of ion-fragments can be obtained per patient. The most relevant features are selected and used to predict the class of the patients. The identity of these selected compounds can be speculated upon by means of the mass spectrum but requires the injection of a chemical standard and comparison of the retention time and fragmentation pattern for proof.
Several programs are available to perform some of the before mentioned steps but most software is designed for targeted analysis of known compounds. An excellent program for untargeted metabolomics discovery is XCMS [54] that is available as a R-package and through an online interface. Now, a peak list per sample can be exported and used for statistical analysis; the peaks are now called features or predictor variables (figure 2). It should be noted that one compounds fragments to many ion-fragments, which are all detected and used as features for statistical analysis. This means this method results in multi-collinearity. The alternative, summing the intensities of the ion-fragments at every retention time, is presumably incorrect in complex samples such as breath because we cannot exclude co-elution of multiple compounds at a single retention time. In that case, the concentration of multiple molecules would be incorrectly summed. Maintaining the ion-fragment structure for statistical analysis allows for differentiation in predictive value between the M/Z values and can help in the identification of the co-elution, although identification of such compounds will remains very difficult (see section from feature to VOC)

Feature selection and classification

The questions in breath studies that use GC-MS analysis can be split into the pathophysiological angle: "Which VOCs are associated with this disease" and the clinical angle: "What is the diagnostic value of breath analysis for this disease". To translate the second question to a clinical application, it is recommendable to obtain a concise list of biomarkers that can be detected with targeted assays in validation trials. Therefore, identification of the few key-biomarkers is important in GC-MS research. Additional reasons for reduction of the number of predictor variables are to increase prediction accuracy by limiting the variance (but potentially increasing bias) and to allow for interpretation of the model.

Several criteria could be applied to select the most appropriate markers. Traditional measures such as p-value and fold-change can describe a breath profile and can be used to show that there are features that are different between the disease states but have a limited value with regard to diagnostic applications. Receiver-operating characteristics analysis can be used to quantify the discrimination between the disease states per
variable and is more closely related to the final application, but still relies on
the simplistic view that the combination of the best individual biomarkers
also gives the best composite signal. Alternatively, several methods are
available that allow for selection using the information of all features. In
logistic regression techniques the Akaine Information Criteria (AIC) are
frequently used to select the most relevant features. However, in breath
research the number of predictor variables is typically an order higher than
the number of subjects and this may limit the use of the AIC. Alternatives
are LASSO and sparse-partial least squares (SPLS) analysis [55]. Both
methods allow for selection of the most relevant features even when the
number of predictors is larger than the number of patients. Importantly,
whatever method is chosen, a high number of predictor variables per
number of included patients frequently induces over fitting (in other
words bias) and thus cross-validation is essential to tune the settings
for the model and estimate the discrimination accuracy. Furthermore, it
should be noted that external validation of the diagnostic accuracy of the
selected features is very important, especially in metabolomic discovery
studies [56-58].

From feature to VOC

Statistical analysis has led to the selection of the most relevant features
and the evaluation of their discrimination accuracy. In order to identify
the VOC that resulted in those ion-fragments the raw GC-MS data should
be consulted. But first, it is important to verify that all ion-fragments with
that retention time show the same association with the disease state as
the selected ion-fragment; this supports the hypothesis that it is indeed
one VOC that eludes at that retention time. If not, co-elution of multiple
compounds is highly suspected and identification will be considerably
more difficult. In the raw GC-MS data, deconvolution software can be
used to obtain as pure mass-spectra as possible of the target compounds.
These spectra can than be compared to reference libraries, such as that of
the National Institute of Standard and Technology (NIST) (figure 2). This
method will lead to a short list of VOCs that have a high matching factor
and to tentative identification of the compound.

However, for most molecules it is impossible to obtain certainty about the
molecular identity, as isomers are frequently difficult to distinguish by
mass-spectra alone. To confirm the identification, pure standard of the compounds that are on the short list should be injected into the column and the molecule of interest will have the exact same retention time as the identified ion-fragment. (figure 2) Incidentally, two isomers may have the same retention time because of insufficient chromatographic separation and thus the conclusion should be that one of the two, or both compounds are different between the target conditions. Identification completes the GC-MS evaluation for breath biomarker discovery but it should be noted that the process of identifying the biochemical pathways that lead to the formation of the identified VOCs in disease is now just starting.

Electronic nose analysis

Electronic noses (eNose), named after their similarities with mammalian olfactory system [11], integratively capture complex VOC mixtures using an array of different sensors [11]. Sensors can be utilized through two mutually exclusive routes: (1) very specific sensors that follow a “lock-and-key” principle that have a very high sensitivity and specificity or (2) semi-selective, cross-reactive sensors that are less sensitive and less selective but an array of which can be used to characterize unknown complex samples [59]. Mamallians rely on a semi-selective “sensor system” and are able to classify approximately 1 trillion different olfactory stimuli [60]. Since the VOCs that should be detected to classify disease most accurately are unknown and most biological samples are highly complex, that approach is used currently most frequently used.

Metal oxides, conducting polymers, optical and infra–red spectroscopy have been used as sensors. Peaks and intensities obtained by mass-spectrometry can also be presented to pattern–recognition algorithms, hereby virtually converting every detected mass into a “sensor” [61]. The composite signal of all sensors in an array can be analyzed using pattern–recognition algorithms. The composite signal of eNose analysis results in a unique fingerprint. Subsequently, these fingerprints can be used for diagnostic and monitoring purposes [61] (figure 3). It should be noted that eNose technology is not designed to tell something about the details of the molecular composition of a sample but is used as a pragmatic method for clinical classification, based on quantitative, probabilistic evidence. In other words, the eNose may be used to confirm or exclude diagnoses rapidly
and non-invasively but without providing the doctor with information on what molecular criteria this diagnoses is based. Therefore, the eNose can be seen as the modern exemplification of empirical medicine. Because the patho-physiological background of a well-understood biochemical assay is ignored with the eNose approach, it is even more important that the training and validation in biomedical research is as rigorous as possible.

**Figure 3:** Electronic nose

*Breath analysis by means of eNose. From the upper left, clockwise: semi-selective sensors react with the VOCs in the breath. The properties of the sensor change as the VOCs bind (e.g. the electrical conductance changes) and that can be measured. All sensors in the array have a different functional layer and react differently to the composition of the breath. Most commonly, the proportion of most variance is captured by dimension reduction with for example principal component analysis. These composite variables can consequently be used for classification.*

The statistical analysis of electronic nose signals is closely related to that described in the section on GC-MS. However with eNoses it is less important to perform feature selection because no molecular information can be obtained from the individual sensors and thus it does not add to the interpretability of the algorithm. Several types of statistical methods have been used in the literature [62], but most involve a form of dimension reduction followed by a classification algorithm (figure 3). Unsupervised dimension reduction methods such as principal component analysis
are frequently used [63], but are easily disturbed by, for example, co-
morbidities to capture other large variations in the data than the disease
of interest [64]. Therefore, other groups have relied on supervised
methods for dimension reduction and classification [65], which can be
combined in partial least square regression [66]. The major advantages
of both principal component analysis and partial least square regression is
that they perform well when the predictor matrix has more variables than
observation (hence the dimension reduction) and when there is multi-
collinearity (as with semi-selective sensors) by reducing the predictor
matrix into a projection of variance. As with GC-MS data, over fitting of
the model to the training data is easy and cross-validation should always
be performed. External validation is eventually required, especially in
clinical studies where disease diagnosis is the main objective.

Current electronic nose technology thus relies on semi-selective sensors,
pattern recognition and empirical disease classification. However, tailored
sensors that have chemical interactions with pre-specified VOCs following
a "lock-and-key" principle could set the stage for different approaches
[59]. If gas-chromatography and mass-spectrometry would allow for
the selection of a limited panel of volatile biological markers, eNose
technology might be used for rapid bedside detection. In this way,
hybrid form eNoses could be constructed that do allow for "black box"
empirical classification but also provide specific information concerning
the biochemical background. Such a combined approach may result in
the integration of the best of both worlds, within a device that still can be
used for disease recognition at the bedside and for continuous monitoring
in ventilated patients.

Conclusion

Breath analysis has shifted from detection of abundantly present molecules
like carbon dioxide to monitoring of scanty volatile organic compounds,
and from a focus on single molecules to a broader look at mixtures of
biological markers. Breath analysis is now used to detect hundreds of
exhaled metabolites, combinations of which have the potential to serve
as composite markers of physiological and pathophysiological processes
as they may reflect oxidative stress, inflammation, infection, and organ
failure. While gas–chromatography and mass–spectrometry is used for
discovery of volatile biological marker, this technique is impractical for daily practice since it is time-consuming and expensive. New techniques such as electronic noses may, guided by gas-chromatography and mass-spectrometry discovery of volatile biomarkers, result in bedside, rapid diagnosis or monitoring of disease by a simple exhalation. Besides applications in the first line and on hospital wards, this may allow for continuous real-time monitoring in critically ill patients, especially when ventilated. For the first time, this may provide in a monitoring system for cellular and molecular processes like organ injury and infection that can momentarily only be extrapolated from alterations in macroscopic physiology.

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Part II

Diagnosis of Acute Respiratory Distress Syndrome
Chapter 3.

A simplified method for exhaled breath collection in intubated and ventilated ICU-patients

Respiratory Physiology and Neurobiology

Abstract

Volatile organic compounds (VOCs) in breath may serve as biomarkers of pulmonary infection or inflammation. We developed and validated a new breath sampling method for VOC analysis in ventilated patients.

Breath was collected from the ventilatory circuit using cheap disposables. VOCs were identified by gas-chromatography and mass-spectrometry (GC-MS) at various minute volumes during ventilation of an artificial lung (in-vitro) and ventilated patients (in-vivo).

Sixty four VOCs emended from the ventilator and tubing. Their concentrations had an inverse correlation with minute volume in in-vitro experiments (median correlation coefficient: –0.61 [25th–75th percentile: –0.66 to –0.43]). Forty four of these “ventilator-associated VOCs” were also observed in-vivo, without correlations with minute volume. In-vivo experiments showed that only positive end–expiratory pressure influenced the concentration of breath VOCs. The sampling method was highly reproducible (median intra–class correlation 0.95 [25th–75th percentile: 0.87-0.97]).

In conclusion, a novel, simple and repeatable sampling method was developed and validated for capturing exhaled VOCs in ventilated patients, which could allow for large-scale breath analysis in clinical studies.
Introduction

Analysis of expired air may be an attractive approach in disease recognition as breath contains metabolites from both pulmonary and systemic pathophysiologic processes [1]. Intubated and mechanically ventilated patients are prone to pulmonary infection and injury [2-4]. Both attribute to changes in volatile organic compounds that can be detected in the exhaled breath [5-10]. Recently, we performed an animal experiment in which we showed that exhaled breath analysis can be used to monitor the development of lung injury in rats that were infused with lipopolysaccharide [11]. Therefore, exhaled breath analysis could allow for real-time bedside diagnosis in critically ill ICU-patients.

Gas-chromatography mass-spectrometry (GC-MS) is gold standard for VOC separation, quantification and identification [12, 13]. However, GC-MS is not available at the bedside. Advancements in sensor technology and rapid mass-spectrometry may allow for a bedside test and even continuous monitoring of ventilated patients in the near future [10, 14, 15]. To facilitate clinical application of these analytical techniques, sample collection should be adapted for bedside use as well. Currently, sample collection from the ventilator circuit is difficult, requiring usage of non-disposable materials, such as sensors, mass flow controllers, tedlar bags and glass syringes [16, 17]. Alternatively, breath can be manually extracted from the ventilator circuit in a glass-syringe but this is prone to errors, labor intensive and cannot be combined with continuous analysis [17, 18]. It is also possible to obtain alveolar air through a suction tube [19]. This method results in loss of airway pressure, which can induce atelectasis and subsequent respiratory failure [20]. Thus sample collection needs to be simplified with the use of disposable materials to allow for large clinical trials using novel analytical techniques.

In contrast to the controlled setting of an animal experiment, several confounding factors should be considered for sample collection in ventilated patients: contamination with VOCs from ventilator and tubing, the influence of ventilator settings (as these cannot be set to standard values due to the severity of illness) and repeatability. Therefore, the aim of this study was to develop and validate a simple method to sample VOCs in the breath of critically ill intubated and mechanically ventilated ICU-
patients. We hypothesized that this method (a) can exclude contaminants from inspired air, ventilator or tubing, (b) account for the influences of the ventilator settings and (c) is repeatable.

Methods

Design and subjects

The study had three parts. First, we performed an in–vitro study of contaminating VOCs in the inspired air. Second, we examined VOCs coming from the ventilatory circuit at various ventilator settings in absence of a patient. And third, a study (in–vivo) was carried out of VOCs in the circuit during mechanical ventilation of intubated patients at the ICU of the Academic Medical Center, Amsterdam, The Netherlands. This 40–beds ICU serves as tertiary reference for both medical and surgical patients. Exhaled air from consecutive newly intubated ICU–patients with the exception of postoperative patients after cardiac surgery was collected and analyzed once per day during the first three days of admittance or until death or discharge. A waiver of informed consent was obtained from the Institutional Review Board (IRB: 10.17.0729). This trial was registered at the Dutch trial register (NTR 2750, www.trialregister.nl).

Sampling method

For clinical care, the ventilation circuit was set up as shown in figure 1, and this set–up was not changed for the purpose of the study, besides the insertion of a side–stream connector. A co–axial tubing system was connected (Universal F2 breathing circuit, Medical product service GmbH, Braunfis, Germany) to a mechanical ventilator (Galileo ventilator, Hamilton, Bonaduz, Switzerland or Servo ventilator, Maquet, Rastatt, Germany) and a heat–and–moister exchanger (HME, Medisize, Hillegom, the Netherlands) was placed at the end. For exhaled breath collection, a T–piece connector (T–piece; 22M/22F with swivel, Medisize, Hillegom, the Netherlands) was placed between the HME and the swivel (Catheter mount, Medisize, Hillegom, the Netherlands). The swivel was connected to the endotracheal tube (Ruschelit safety clear plus, Teleflex medical, Athlone, Ireland), which is in direct contact with the upper part of the lower airways of the patient. To produce a side stream flow, the T–piece was mounted with 50 cm bubbling tube (Bubble tubing PHS3/30G 3x5mm
30m, Medisize, Vantaa, Finland), which was locked with a three-way stop-cock before insertion into the ventilatory circuit. Air was adsorbed on a stainless steel tube (6mm O.D * 7 inch, Supelco, Zwijndrecht, The Netherlands) filled with Tenax GR (250 mg/tube, Varian Chrompack, Middelburg, The Netherlands) using a fixed flow of 200 mL/min for 10 minutes. Two liters of mixed air corresponds to 1.3 liters of expiratory air, after adjustment for an inspiratory:expiratory-ratio of 1:2. The flow was generated by a membrane pump (XaviTech V200 GAS 3.2 – 26V DC, Harnosand, Sweden) and controlled by means of a flow controller (MEMS flow sensor D6F-P0010A, Omron, Hoofddorp, the Netherlands). The samples were stored for a maximum of two weeks at 4°C.

**Experimental protocol**

*Contaminants from inspired air and ventilator or tubing*

Contamination of the sample by VOCs from (a) the nitrogen–oxygen mixture (medical grade air) used for mechanical ventilation and (b) the ventilator and tubing were investigated *in-vitro*. Two liters of air was sampled from the hospitals' compressed air source. The ventilation circuit was set up as if a patient was ventilated (figure 1, top part). For this experiment, instead of the lungs of the patients, a Tedlar-bag was ventilated. Furthermore, the VOCs released by the side stream connector were investigated separately by purging the side stream with 2 liters of pure nitrogen gas.

*Influences of the ventilator settings*

To investigate the influence of ventilator settings, an artificial lung (VT Plus HF, Fluke Adquipment Medical, Hellevoetssluis, The Netherlands) was ventilated using the above described set-up (figure 1, top part). A wide range of diverse clinically relevant ventilator settings was investigated: minute ventilation (MV) ranged from: 4.5 – 17 L/min, positive end expiratory pressure (PEEP) ranged from: 5 – 15 cm H₂O and maximal inspiratory pressure (Pmax) ranged from: 10 – 45 cm H₂O.
Figure 1: Schematic representation of the sampling method.

Air is collected through a T-piece distal of the endotracheal tube, proximal of the heat and moisture exchanger. To test the VOCs released by the system, a Tedlar bag was connected for air collection. 2 Liters of air is collected onto a Tenax tube with a flow of 200 ml/min for GC–MS analysis. To test the influence of positive end-expiratory pressure, minute ventilation and maximal airway pressure, an artificial lung was connected to the tubing. The side stream connector and tubing was also investigated separately. The typical flow (minute ventilation) in the ventilator, tubing and endotracheal tube is 4 to 17 liter/minute while the flow in the side stream connector to the tenax tube is always 200 ml/min.

Repeatability of breath collection

Three subsequent samples were collected per time point, per included patient (figure 1, bottom part). The three samples were taken within a 30-minute timeframe.
**GC–MS analysis**

GC–MS analysis was performed as described previously [8]. In brief, tubes were thermally desorbed using helium inside a thermal desorption unit (Gerstel, Mülheim an der Ruhr, Germany). The sample was transferred to a packed liner, heated to 300 °C for three minutes and subsequently relocated onto a cold trap filled with Tenax TA (at –150 °C), which was heated after two minutes to 280 °C at 20 °C/sec and splitless injected onto the chromatographic column. Compounds were separated using capillary gas–chromatography with helium as a carrier gas at 1.2 mL/min (6890N GC, Agilent, Santa Clara, CA, USA) on a VF1–MS column (30 m * 0.25 mm, film thickness 1 µm, 100% dimethylpolysiloxane, Varian Chrompack, Middelburg, The Netherlands). The temperature of the gas chromatograph was adjusted in three steps: 40°C for 5 minutes, increased until 300°C with 10°C/minute, held isothermal for 5 minutes. The first 15 minutes of data acquisition were used for statistical analysis. A quadrupole mass spectrometer (5975 MSD, Agilent, Santa Clara, CA, USA), in electron impact ionization mode at 70 eV, was used for the detection of product ions (ranging from 29 – 450 Da).

Calibration standards were produced with a home built dilution system using 10 ppmv toluene in nitrogen (Air Products, Amsterdam, The Netherlands). The sensitivity and linear range of the system were checked by loading toluene standards in the range of 13 – 200 ng on a Tenax adsorption tube. The calibration curve was linear in this range (r^2 = 0.9998). The LOD was 0.9 ng and the LLOQ was 0.23 ng. Peak–detection and alignment were performed using the Xcms–package for R [21] (Stripps center for metabolomics, La Jolla, CA, USA) and resulted in an ion–fragment peak table as input for statistical analysis. Ion–fragments were grouped based on retention time and correlation coefficients categories that represent VOCs. Fragmented ions were manually checked in the raw chromatograms and the corresponding metabolites were tentatively identified based on NIST–library matching. When this procedure did not result in identification, the compound was called unidentified.
Data analysis

Data–analyses were performed in R (v2.14, www.r-project.org) using the R–studio interface. P–values below 0.05 were considered significant. Normal distribution was investigated using histograms and QQ–plots and the performed tests were adapted accordingly.

Data analysis was performed in accordance to the final two aims described in the introduction: the influence of ventilator settings (aim b) and the repeatability (aim c). Firstly, concentrations of VOCs were associated with MV, Pmax and PEEP using Pearson’s correlation for ventilator and tubing data alone. This procedure was repeated for the patient data. Confounding and interaction between ventilator settings was checked by multi–variable linear regression. Intra–class correlation (ICC) was used to quantify the difference between inter– and intra–patient variance. This method was repeated using random group allocation to check for false–discovery.

Results

Patients

Twenty–six ICU–patients were included in the study between November 2011 and February 2012. The baseline characteristics are listed in table 1. The included population was severely ill as illustrated by high Acute Physiology and Chronic Health Evaluation (APACHE) II scores and Simplified Acute Physiology Scores (SAPS). More than half the patients had sepsis at admission to the ICU and approximately one–third suffered from acute respiratory distress syndrome (ARDS) [22]. The severity of illness was also reflected by a high ICU–mortality rate. Thirty–five measurements were performed in triplo during the first 72 hours of ICU–admission under highly variable circumstances (table 2).
Table 1: Patient Characteristics.

<table>
<thead>
<tr>
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<th>N = 26</th>
</tr>
</thead>
<tbody>
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<td>Age</td>
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</tr>
<tr>
<td>Male</td>
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</tr>
<tr>
<td>APACHE II</td>
<td>21 (19–26)</td>
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<tr>
<td>SAPS II</td>
<td>58 (43–70)</td>
</tr>
<tr>
<td>Admission type</td>
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</tr>
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<tr>
<td>Surgical</td>
<td>9 (34.6)</td>
</tr>
<tr>
<td>Pulmonary edema</td>
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</tr>
<tr>
<td>ARDS</td>
<td>7 (26.9)</td>
</tr>
<tr>
<td>CPE</td>
<td>3 (11.5)</td>
</tr>
<tr>
<td>No</td>
<td>16 (61.5)</td>
</tr>
<tr>
<td>Sepsis</td>
<td>16 (61.5)</td>
</tr>
<tr>
<td>Died in the ICU</td>
<td>4 (15.4)</td>
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Table 2: Measurement Characteristics.

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<td>Day after admission</td>
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<td>0</td>
<td>25 (71.4)</td>
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<tr>
<td>1</td>
<td>7 (20)</td>
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<tr>
<td>2</td>
<td>3 (8.6)</td>
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<tr>
<td>Pmax (cm H₂O)</td>
<td>18 (14–29)</td>
</tr>
<tr>
<td>PEEP (cm H₂O)</td>
<td>5 (5–9)</td>
</tr>
<tr>
<td>Vₜ (mL)</td>
<td>463 (378–592)</td>
</tr>
<tr>
<td>Vₜ per kg ideal body weight (mL/kg)</td>
<td>6.3 (5.3–8.7)</td>
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<tr>
<td>Minute volume (L/min)</td>
<td>10.8 (8.6–13.7)</td>
</tr>
<tr>
<td>FiO₂ (%)</td>
<td>40 (35–45)</td>
</tr>
<tr>
<td>PaO₂ (kPa)</td>
<td>13.0 (11.6–15.6)</td>
</tr>
<tr>
<td>PaO₂./FiO₂ (kPa)</td>
<td>264 (199–312)</td>
</tr>
<tr>
<td>PaCO₂ (kPa)</td>
<td>4.9 (4.6–5.6)</td>
</tr>
</tbody>
</table>

Contamination

Fifteen VOCs were detected in medical grade air: acetaldehyde, ethanol, acetonitrile, acetone, dichloromethane, tetrahydrofuran, benzene, an unidentified alcohol, heptane, toluene, hexanal, xylene (any isomer), cyclohexanone, octane and benzaldehyde. All concentrations were around the lower limit of quantification with the exception of acetone (0.4 ng/l)

Influence of ventilator settings in-vitro

Sixty four volatile organic compounds were detected during in-vitro study of the ventilator and tubing (table 3). Most VOCs were found in lower concentrations when the minute volume ventilation increased (median
correlation coefficient: −0.61 [25th−75th percentile: −0.66 to −0.43]). This was similar to increasing Pmax (median correlation coefficient: −0.46 [25th−75th percentile: −0.61 to −0.38]). PEEP was not correlated with VOC concentrations (median correlation coefficient: −0.07 [25th−75th percentile: −0.20 to 0.00]). In multi-variate linear regression MV was most frequently significantly associated with VOC concentrations (38 of 64 VOCs), in contrast to Pmax (17 of 64 VOCs significantly correlated). This phenomenon is illustrated for cyclohexanone in Figure 2.

**Influence of ventilator settings in–vivo**

Forty four volatile metabolites that were detected in the samples coming from the ventilator and tubing (“ventilator-associated” VOCs), were also detected in the exhaled breath of the patients. Thus 20 “ventilator-associated” VOCs were not found in the breath of patients. The correlations of VOCs with MV and Pmax found in the in-vitro experiment were not replicated in breath samples. The median correlation coefficient was 0.00 [25th−75th percentile: −0.09 to 0.16] and 0.14 [25th−75th percentile: 0.04 to 0.22] for MV and Pmax, respectively. In contrast, ventilator and tubing VOCs in exhaled breath showed a moderate, positive correlation with PEEP (median correlation coefficient: 0.21 [25th−75th percentile: 0.10 to 0.29]). 23 ventilator/tubing VOCs (34%) were found in higher and 5 (8%) in lower concentration in breath than in the in–vitro setting (table 3).

Two hundred and nine VOCs, that were not found in the ventilator and tubing were detected in all exhaled breath samples. These VOCs were not correlated with MV (median regression coefficient: −0.01 [25th−75th percentile: −0.10 to 0.10]. Breath VOCs showed a moderate, positive correlation with Pmax and PEEP (median correlation coefficient: 0.13 [25th−75th percentile: 0.05 to 0.20] and 0.29 [25th−75th percentile: 0.14 to 0.38].

**Repeatability**

The median intra–class correlation for exhaled VOCs was 0.95 [25th−75th percentile: 0.87 to 0.97] (figure 3). Re-analysis with a random groups resulted in significantly lower intra–class correlation (median: 3x10−9 [25th−75th percentile: 0 – 0.01], p<0.001).
**Figure 2:** The correlation between cyclohexanone and minute ventilation and maximal inspiratory pressure.

There is a negative correlation between minute volume ventilation and cyclohexanone concentration in in–vitro ventilator samples (top figures), but not in in–vivo breath samples (bottom figures). MV = minute ventilation, Pmax = maximal inspiratory pressure.

**Figure 3:** Histogram of intra–class correlations.

The black bars show the intra–class correlations for all VOCs found in the exhaled breath of intubated and ventilated ICU–patients. The grey bars show this for random group allocation, to check for false–discovery. An ICC of 1 shows that a measurement is perfectly repeatable and of 0 that it is not repeatable at all.
<table>
<thead>
<tr>
<th>Compound</th>
<th>MV</th>
<th>Pmax</th>
<th>Inspired air</th>
<th>Side stream</th>
<th>Breath</th>
<th>Source / Mechanism</th>
<th>Possible marker</th>
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<td>1</td>
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<td>Ventilator and tubing</td>
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<td>Ventilator and tubing</td>
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Table 3: Volatile organic compounds found in ventilator and/or tubing.
### Table 3 (continued): Volatile organic compounds found in ventilator and/or tubing

<table>
<thead>
<tr>
<th>#</th>
<th>Compound</th>
<th>MV</th>
<th>Pmax</th>
<th>Inspired air</th>
<th>Side stream</th>
<th>Breath</th>
<th>Source / Mechanism</th>
<th>Possible marker</th>
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<tr>
<td>27</td>
<td>Formamide, N,N-dimethyl-</td>
<td>-0.67**</td>
<td>-0.45</td>
<td>-</td>
<td>-</td>
<td>↑</td>
<td>Ventilator and tubing / endogenous</td>
<td>+</td>
</tr>
<tr>
<td>28</td>
<td>Toluene</td>
<td>-0.71**</td>
<td>-0.72**</td>
<td>+</td>
<td>↑</td>
<td>↑</td>
<td>Inspired air / side stream connection / possibly endogenous</td>
<td>+</td>
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<tr>
<td>29</td>
<td>2–Pentanone, 4,4-dimethyl–</td>
<td>-0.75**</td>
<td>-0.38</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Ventilator and tubing</td>
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<td>30</td>
<td>Hexanal</td>
<td>-0.43</td>
<td>-0.55</td>
<td>+</td>
<td>↑</td>
<td>↑</td>
<td>Side stream connection / endogenous</td>
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<td>-</td>
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<td>↑</td>
<td>Ventilator and tubing / endogenous</td>
<td>+</td>
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<tr>
<td>32</td>
<td>Acetic acid, butyl ester</td>
<td>-0.65**</td>
<td>-0.66**</td>
<td>-</td>
<td>↓</td>
<td>↑</td>
<td>Ventilator and tubing / endogenous</td>
<td>+</td>
</tr>
<tr>
<td>33</td>
<td>Octane</td>
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<td>-0.09</td>
<td>+</td>
<td>↓</td>
<td>↑</td>
<td>Ventilator and tubing / endogenous</td>
<td>+</td>
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<td>34</td>
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<td>-0.65**</td>
<td>-0.44</td>
<td>-</td>
<td>-</td>
<td>↑</td>
<td>Ventilator and tubing / endogenous</td>
<td>+</td>
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<tr>
<td>35</td>
<td>Furfural</td>
<td>-0.40</td>
<td>-0.60**</td>
<td>-</td>
<td>↓</td>
<td>↑</td>
<td>Ventilator and tubing / endogenous</td>
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<tr>
<td>36</td>
<td>Cyclotrisiloxane, hexamethyl– †</td>
<td>-0.08</td>
<td>-0.45</td>
<td>-</td>
<td>-</td>
<td>↑</td>
<td>Water in breath → ↑ column bleed</td>
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<tr>
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<td>-0.48*</td>
<td>-</td>
<td>↓</td>
<td>↑</td>
<td>Ventilator and tubing / endogenous</td>
<td>+</td>
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<tr>
<td>38</td>
<td>1,3–Cyclopentanedione, 4,4–dimethyl–1–heptene</td>
<td>-0.62**</td>
<td>-0.50*</td>
<td>-</td>
<td>-</td>
<td>≈</td>
<td>Ventilator and tubing</td>
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<tr>
<td>39</td>
<td>2,4–Dimethyl–1–heptene</td>
<td>-0.58*</td>
<td>-0.31</td>
<td>-</td>
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<td>-0.66**</td>
<td>-0.52*</td>
<td>-</td>
<td>↓</td>
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<td>41</td>
<td>Ethylbenzene</td>
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<td>-0.52*</td>
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<td>↓</td>
<td>≈</td>
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<tr>
<td>42</td>
<td>Unidentified</td>
<td>-0.59**</td>
<td>-0.40</td>
<td>-</td>
<td>↓</td>
<td>≈</td>
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<tr>
<td>43</td>
<td>Cyclohexanone</td>
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<td>-0.43</td>
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<td>44</td>
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<td>45</td>
<td>Styrene</td>
<td>-0.66**</td>
<td>-0.47*</td>
<td>-</td>
<td>-</td>
<td>↑</td>
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<td>+</td>
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<tr>
<td>46</td>
<td>Xylene (p or o isomer)</td>
<td>-0.59</td>
<td>-0.45</td>
<td>-</td>
<td>-</td>
<td>≈</td>
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Table 3 (continued): Volatile organic compounds found in ventilator and/or tubing
Discussion

This study describes a simplified methodology for breath collection in intubated and mechanically ventilated ICU–patients. Several VOCs emanated from the ventilator and tubing, some of which were influenced by minute volume ventilation in–vitro albeit not in–vivo. Inspired air contained some VOCs in very low concentrations. Finally, the described method provided repeatable detection of VOCs in the ventilator circuit. This indicates that simple technology added to a standard ICU-setting allows capturing exhaled VOCs in the ventilator circuit of mechanically ventilated patients.

Contamination from inspired air and the ventilator and tubing

To our knowledge, this is the first study to systematically investigate the interference of VOCs from the ventilator and tubing. Sixty four volatile organic compounds were found to be emitted by the ventilator and the tubing. Most of these compounds could also be found in the breath of intubated and mechanically ventilated ICU–patients. The VOCs coming from the ventilator and tubing that were not found or in lower concentration in exhaled breath may be absorbed and metabolized by the patient. One of the detected molecules, cyclohexanone, was previously described to emanate from endotracheal tubes [23, 24]. The other molecules were not previously described is this context. Cyclohexanone was also found in medical grade air, together with fourteen other VOCs, in very low concentrations. This could not explain the higher concentration obtained from the ventilator and the tubing. Contamination of VOCs from the inspiratory air was previously described to be a problem [15, 25]. However, given the very low concentrations of VOCs found in inspiratory air in this study this does not seem to greatly influence the results found in our study. This may partly be due to the use of Tenax GR as a sorbent. Breakthrough may occur during absorption of very volatile organic compounds on Tenax GR, therefore it cannot be excluded that the original concentrations in inspired air were considerably larger [26].

Influence of ventilator settings

Concentrations of several VOCs from the ventilator and tubing were found to inversely correlate with minute ventilation and maximal inspiratory
pressure. This selectivity towards certain volatile molecules may be explained by a two-compartment model in which some VOCs originate from the ventilator, tubing and endotracheal tube and thus are diluted during higher minute ventilation (ranging from 4 to 17 liters/minute) whereas VOCs from the side stream are constantly sampled at 200 ml/min (see figure 1). This seems to apply to most VOCs described in table 3 (higher correlation coefficient if not found in side stream), but some VOCs behaved differently. For example, octane was present in lower concentrations in the side stream than in the ventilator and tubing samples. Therefore, it is very likely to be emitted from the central tubing and should be influenced by minute volume ventilation, but we did not observe this. Of note, octane was found in relatively high concentrations in the exhaled breath, suggesting an additive endogenous origin of this compound.

The correlation between minute volume ventilation and the VOC concentration was not reproduced in the in-vivo data. Some of the “ventilator VOCs” were found in higher concentrations in the patient breath. This indicated that these compounds are also produced in the human body. It may be that this additive production conceals the influence of ventilator settings on emission of VOCs from the ventilator and the tubing. In contrast to MV, Pmax and PEEP were positively correlated with VOC concentrations in the breath of intubated and mechanically ventilated patients. This is likely the result of ventilatory standard in clinical care. In our hospital, PEEP is namely increased per protocol in patients with impaired oxygenation and the inspiratory pressure (which affects Pmax) is adjusted to maintain a tidal volume of 6ml/kg. This means that patients with a decreased compliance of the respiratory system and problems with oxygenation will have a higher PEEP and Pmax. Therefore, the correlation between ventilation pressures and VOCs is possibly a reflection of severity of pulmonary injury [27]. This phenomenon is also biologically plausible as many VOCs are connected to oxidative stress reactions, which is one of the mechanisms that mediates pulmonary injury [11].

**Repeatability**

The sampling method showed to be repeatable based on high intra-class correlation. Previous papers on collection of exhaled breath in intubated
and mechanically ventilated ICU-patients did not report the intra–class correlation as measure for repeatability. Filipiak et al. used the relative standard deviation in a recent, excellent paper [17] and showed that their method was highly repeatable under controlled circumstances. In contrast to the relative standard deviation, the intra–class correlation summarizes both between patient–variation as within patient–variation and is therefore preferred to assess repeatability [28]. To our knowledge, this is thus the first paper to adequately assess repeatability of VOC detection in breath of ventilated ICU–patients.

Limitations

We tried our utmost to standardize the experimental settings. Nevertheless, our study may have some limitations. One of the potential weaknesses of this study is that alveolar air was not selectively sampled, as recommended [29], but a mixed sample was obtained. Alveolar air contains the highest concentrations of systemically produced volatile organic compounds and the lowest concentration of exogenous molecules [30, 31]. However, the sampling method described in this study was not designed for the analysis of systemically produced VOCs per se but rather for VOCs produced locally in the whole lung, which includes the airways. The conducting airways are increasingly recognized as an active compartment in the exchange of more soluble gasses [32]. Levels of biological markers of inflammation and coagulation pathways are altered in lavage fluid obtained from the bronchial tree in patients with pulmonary infection or injury, thus metabolic changes might also emerge in these parts of the lung [33-36].

Another limitation of this method is the collection of mixed inspiratory/ expiratory air. We chose to do so, based on practical and safety reasons, allowing the usage of disposable materials. An alternative is to remove the HME and place the connector in the expiratory circuit [5, 37, 38]. However, prolonged removal of the HME is not without risks and is contraindicated in patients with respiratory failure (e.g., acute respiratory distress syndrome) [39]. It should be noted that the proposed sampling method was adapted for mechanically ventilated patients only and cannot be extended to spontaneous breathing individuals.
Safety and costs

No adverse events (desaturation and changes in tidal volumes or pressures) were reported during 105 sample collections, while the included patients were severely ill. Some of the patients sampled had such compromised respiratory function that a diagnostic broncho–alveolar lavage could not be performed at the time of breath collection. Furthermore, the set–up was very low costs (€ 1.20 for disposable sampling equipment) and easy to use. A student could be trained to perform sample collection following a “see one, do one, teach one”–principle. However, the analysis of the samples using GC–MS was not low costs. In this study, GC–MS was used as a tool to detect VOCs because of the high sensitivity towards low abundance compounds of this technology. The purchase and maintenance of a thermal desorption GC–MS is expensive. Furthermore, a highly trained technician is needed to perform the analyses. It should be noted that this study did not aim to describe a new method for VOC–analysis by GC–MS but rather a novel method for sample collection that can be used for future bedside technologies. The chosen materials and settings for the thermal desorption (adsorption material and amount, injection method), chromatography (column and temperature protocol) and mass spectrometry (quadrupole or time–of–flight) are therefore arbitrary and not the focus of this paper. Adsorption material and chromatographic column can be adapted per study for specific aims. In clinical practice sample analysis using GC–MS should ideally be replaced by cheaper alternatives (e.g. sensor based technology, ion–mobility spectroscopy or proton–ion reaction mass spectrometry).

Implications

There is a growing need for large diagnostic studies using non–invasive technologies in critically ill patients. Breath analysis could in the near future provide a rapid, cheap and easy test in mechanically ventilated ICU–patients. The first step to a clinical test is identifying the volatile organic compounds altered in respiratory infection and inflammation using GC–MS [5-10]. The sampling method described in this study could allow for application of exhaled breath analysis in large clinical studies. Importantly, our data suggest that we can exclude VOCs that are emitted by the ventilator and the tubing alone while keeping the possibility to
include endogenously produced VOCs. GC–MS may not be the ideal technique for VOC detection as it is not widely available, expensive and time consuming thereby limiting its potential for clinical intensive care practice. Future technological innovations in the field of breath research should consequently focus on the development of nanotechnology for rapid detection of volatile organic compounds found in GC–MS–driven research [40, 41]. These trials should now be designed and performed, strictly following STARD–guidelines for testing diagnostic accuracy [42].

Conclusions

In conclusion, a novel, simple and repeatable sampling method was developed and validated for capturing exhaled volatile organic compounds in intubated, mechanically ventilated ICU–patients during standard care. This sampling method allows for breath testing in larger clinical studies to identify diagnostic volatile organic compounds for infection and inflammation of the lung, preferably using sensor based technology for VOC detection.

References

Pulmonary Inflammatory Response. Journal of Infectious Diseases 159: 189-194


Chapter 4.

Exhaled Breath Profiling for diagnosing Acute Respiratory Distress Syndrome

*BMC Pulmonary Medicine 2014*

Lieuwe DJ Bos, Marcus J Schultz & Peter J Sterk
Abstract

The acute respiratory distress syndrome (ARDS) is a common, devastating complication of critical illness that is characterized by pulmonary injury and inflammation. The clinical diagnosis may be improved by means of objective biological markers. Electronic nose (eNose) technology can rapidly and non-invasively provide breath prints, which are profiles of volatile metabolites in the exhaled breath. We hypothesized that breath prints could facilitate accurate diagnosis of ARDS in intubated and ventilated intensive care unit (ICU) patients.

Prospective single-center cohort study with training and temporal external validation cohort. Breath of newly intubated and mechanically ventilated ICU-patients was analyzed using an electronic nose within 24 hours after admission. ARDS was diagnosed and classified by the Berlin clinical consensus definition. The eNose was trained to recognize ARDS in a training cohort and the diagnostic performance was evaluated in a temporal external validation cohort.

In the training cohort (40 patients with ARDS versus 66 controls) the diagnostic model for ARDS showed a moderate discrimination, with an area under the receiver–operator characteristic curve (AUC–ROC) of 0.72 (95%–confidence interval (CI): 0.63-0.82). In the external validation cohort (18 patients with ARDS versus 26 controls) the AUC–ROC was 0.71 [95%–CI: 0.54 – 0.87]. Restricting discrimination to patients with moderate or severe ARDS versus controls resulted in an AUC–ROC of 0.80 [95%–CI: 0.70 – 0.90]. The exhaled breath profile from patients with cardiopulmonary edema and pneumonia was different from that of patients with moderate/severe ARDS.

An electronic nose can rapidly and non-invasively discriminate between patients with and without ARDS with modest accuracy. Diagnostic accuracy increased when only moderate and severe ARDS patients were considered.
Background

The acute respiratory distress syndrome is a common, devastating complication of critical illness that is characterized by bilateral protein rich pulmonary edema due to injury and inflammation of the lung. A valid and reliable diagnosis of ARDS is considered essential for clinical management and to facilitate enrolment of consistent patient phenotypes into clinical trials [1]. Presently, a new and improved consensus definition of ARDS is used that is based on clinical, radiological and physiological criteria [1]. These criteria are highly suitable for epidemiological studies but only show a moderate correlation with post–mortem pathological findings [2]. ARDS can be mistaken for pneumonia (uni–lateral edema, infection and inflammation) or cardiogenic pulmonary edema (CPE) (low–protein edema due to hydrostatic pressure), and vice versa [2, 3]. Thus, there is need for objective markers to group phenotypes more consistently [4].

Use of biological markers could improve the diagnostic process of ARDS since such markers may change before the clinical criteria of ARDS are met [5]. It can be argued that biological markers from lung tissue contain more relevant biochemical information for ARDS diagnosis than plasma markers [6-9]. Exhaled breath contains hundreds of volatile organic compounds (VOCs) that are produced with diverse infectious and inflammatory processes, both in the lung and elsewhere in the body [10-15]. Previous studies of biological markers in the breath of critically ill patients focussed on exhaled breath condensate [16-20]. However, direct analysis of volatile metabolites in the gas phase is also available now [21, 22]. This has many advantages, as samples do not require extensive pre–processing, analysis is rapid and may be performed continuously using novel technologies [23].

We hypothesized that VOCs could be used to accurately diagnose and classify ARDS in intubated and ventilated intensive care unit (ICU) patients. The secondary objectives were to investigate the influence of ARDS severity and the underlying causal factor (i.e., pulmonary or non–pulmonary) on diagnostic accuracy. Thirdly, we aimed to investigate the classification of uncomplicated pneumonia and CPE by exhaled breath analysis. Here we focus on exhaled breath profiling (so–called ‘breath prints’) using a electronic Nose (eNose) technology that relies on cross–reactive sensors, meaning that each sensor is responsive to a variety of
VOCs [24, 25].

**Methods**

*Design, subjects and settings*

This was a prospective single centre cohort study. All patients admitted to the ICU, with the exception of cardiopulmonary surgery patients, were screened. The only inclusion criterion was mechanical ventilation within the first 24 hours of ICU-admission. Exclusion criteria were (1) previous ICU admission or mechanical ventilation, (2) logistic problems or (3) explicit objection to research by the family.

*Ethical approval and informed consent*

The institutional review board of the Academic Medical Center, Amsterdam, The Netherlands, decided that the study did not fulfil all criteria for medical research as stated in the Dutch ‘law on medical research’ because of the non-invasiveness and absence of burden of examining exhaled air (IRB: 10.17.0729). It was judged that exhaled breath could be analyzed without informed consent of the patient. This trial was registered at the Dutch Trial Register (NTR 2750, www.trialregister.nl).

*Training and validation cohort*

The present study strictly adhered to the 25 required items of STARD-guidelines on the investigation of diagnostic accuracy (eTable 1) [26]. During three inclusion periods of ~ 3 months, between January 2011 and February 2012, newly admitted ICU–patients were screened during weekdays. Patients included in the first 2 periods were used in the training cohort; patients included in the last period served as a temporal external validation cohort [27].

*Sample size calculation*

Based on a pilot study the estimated sensitivity of exhaled breath profiling for discriminating the two extremes, definite ARDS and definite control patients, was 96.5% [28]. Assuming a prevalence of 50%, an alpha of 0.05 and a 95% confidence interval, the predicted sample size was 104 for the training cohort [29]. A validation cohort half the size of the training cohort was included, according to recommendations on design and analysis of metabolomics studies [30].
Clinical diagnosis of ARDS

A team of trained clinical research fellows prospectively scored the presence of ARDS [31], which was later re-evaluated according to the new Berlin definition that included the separation in mild, moderate and severe ARDS [1]. Importantly, the assessors were always blind for the eNose signal. All observers were trained on several occasions before the start of the study. All assessors had attended meetings in which clinical case vignettes were discussed and had at least 6 months of work experience [32].

Competing diagnoses

The diagnosis of community- or hospital-acquired pneumonia consisted of adapted Center for Disease Control-criteria and a post-hoc likelihood of infection was scored (none, possible, probable or proven; see eTable 2) [32, 33]. In contrast to ARDS, the diagnosis of CPE required that the findings (acute onset, bilateral infiltrates and PaO2/FiO2 ratio < 300) were fully explained by cardiac dysfunction based on echocardiography [1].

Exhaled breath profiling

Existing methodology [21] was adapted for the specific situation of breath collection in intubated and ventilated ICU-patients, as reported previously (figure 1, upper part) [34]. A co-axial tubing system was connected (Universal F2 breathing circuit, Medical product service GmbH, Braunfis, Germany) to a mechanical ventilator (Galileo ventilator, Hamilton, Bonaduz, Switzerland or Servo ventilator, Maquet, Rastatt, Germany) and a heat–moist exchanger (HME, Medisize, Hillegom, the Netherlands) was placed at the end as part of routine practice. A T-piece connector (T-piece; 22M/22F with swivel, Medisize, Hillegom, the Netherlands) was placed between the HME and the swivel (Catheter mount, Medisize, Hillegom, the Netherlands). The swivel was connected to the endotracheal tube (Ruschelit safety clear plus, Teleflex medical, Athlone, Ireland). To produce a side-stream flow, the T-piece was mounted with 50 cm bubbling tube (Bubble tubing PHS3/30G 3x5mm 30m, Medisize, Vantaa, Finland), which was locked with a three-way stop-cock before insertion into the ventilatory circuit. Exhaled breath was collected (approximately 50 ml/min for 1 minute) and led to a portable eNose, the Cyranose 320 (Smith Detections, Pasadena, CA), containing a nano-composite sensor array with 32 polymer sensors. These sensors swell as volatile organic
compounds diffuse into the polymer thereby causing a change in the electrical resistance. The relative change in electrical resistance is saved onto an onboard memory and can later be copied to an offline database. A baseline measurement was performed for 30 seconds through a VOC–filter type A1 (North Safety, Middelburg, the Netherlands). Thereafter, exhaled air was collected and analyzed on line for 60 seconds, using two separate Cyranose eNoses. This procedure was repeated. Data from every initial measurement was disregarded in the analysis because of deviant raw data, as recommended by the manufacturer [21]. The index test and reference test were always performed on the same day, within 24 hours after admission and were blinded for each other.

Sensor drift over time
We determined sensor drift over time [35]. Per inclusion period, this shift was assumed to be linear. Sensor data was corrected for drift over time, per period, by transformation into standardized residuals by linear regression. This is similar to multiplicative correction, but without the usage of a chemical standard [36].

Group allocation
ARDS patients were classified as cases and used to train and validate a diagnostic algorithm. Control patients did not fulfil the criteria for ARDS, but could have infiltrates on chest radiography or oxygenation problems, and had no or a low likelihood of having pneumonia or CPE (e.g. a patients with interstitial lung disease could be in the control group). The trained algorithm was used to predict the probability of group membership in the patients with competing diagnoses (pneumonia and CPE).

Statistical analysis
Differences between the groups were compared using the Mann–Whitney U or Kruskal–Wallis test for continuous variables and chi–square for categorical variables. Data was summarized using the median and 25–75th percentile for continuous variables and with count and percentage for categorical variables. All analyses were performed in R statistics using the R–studio interface [37]. P–values below 0.05 were considered significant.
Exhaled breath was sampled and analyzed using an electronic nose with a side-stream connection distal from the endotracheal tube. This resulted in a response for the 32 polymer sensors in the nano–composite sensor array. The eNose was trained using sparse–partial least square (SPLS) logistic regression with 10,000–fold cross-validation. Data from the training cohort was split into a fraction for model building and model evaluation (10 cases and 10 controls). The algorithm that provided the best internally validated diagnostic accuracy, evaluated by the area under the receiver operating characteristics curve (ROC–AUC), was selected for blind testing in the validation cohort and the ROC–AUC with optimal sensitivity and specificity was reported. Differences in the predictive algorithm between different subgroups (severity of disease, pulmonary and non–pulmonary ARDS) were analyzed using non–parametric tests and the ROC–AUC was reported. Furthermore, the ROC–AUC for distinguishing CPE and pneumonia from ARDS and moderate/severe ARDS only was calculated. A sensitivity analysis was also performed.
The eNose was trained using sparse–partial least square (SPLS) logistic regression with 10,000–fold cross–validation. SPLS analysis is a form of regression that can select predictive variables and limit false discovery in situations were large number of independent variables are investigated in low numbers of individuals [38]. Data from the training cohort was split into a fraction for model building and model evaluation (10 cases and 10 controls). The algorithm that provided the best, robust internally validated diagnostic accuracy, evaluated by the area under the receiver operating characteristics curve (ROC-AUC), was selected for blind testing in the validation cohort and the ROC-AUC with optimal sensitivity and specificity was reported. The process of temporal external validation is required to assess the actual diagnostic accuracy of the eNose for ARDS [30]. To check for over–fitting of the algorithm, the previous steps were 1000 times repeated with permutated group allocation.

Differences in the predictive algorithm between different subgroups (severity of disease, pulmonary and non–pulmonary ARDS) were analyzed using non–parametric tests and the ROC-AUC was reported. Furthermore, the ROC-AUC for distinguishing CPE and pneumonia from ARDS and moderate/severe ARDS only was calculated. A sensitivity analysis was performed using logistic regression on comorbidities that are known to influence breath prints (chronic pulmonary disease and cancer, see table 1), the PaO2/FiO2 ratio, minute volume ventilation and measures of severity of disease (Acute Physiology and Chronic Health Evaluation (APACHE) II and Simplified Acute Physiology Score (SAPS) II).

Results

Subjects

Six hundred twenty–one patients were screened, of whom 274 were not eligible and 120 met exclusion criteria (see figure 2). Thus, 207 patients were included. Exhaled breath profiles were not obtained because of technical problems in 27 patients, leaving 180 patients for analysis. No adverse events were reported during or shortly after breath collection. Fifty–eight (32%) patients fulfilled the definition for ARDS [1], 35 patients were classified as having mild ARDS, and 22 and 1 patient as moderate and severe ARDS, respectively. 92 (51%) patients did not fulfil the definition for ARDS; these patients served as control patients. Competing
diagnoses were pneumonia (11 patients) and CPE (19 patients). None of the control patients progressed towards ARDS during the first three days of ICU–admission. Table 1 shows baseline characteristics and respiratory parameters.

**Figure 2:** Patient inclusion

Sensor drift

The sensor signal of the eNoses demonstrated drift over the three periods and within the second period (eFigure 1–A). After transformation into standardized residuals by linear regression, these trends disappeared (eFigure 1–B).

Training and internal validation

SPLS logistic regression resulted in the selection of 7 sensors (sensors 4, 8, 9, 11, 16, 28 and 30), the regression coefficients of which can be found in eTable 3. The AUC–ROC for ARDS in the model development cohort was 0.73 (95%–confidence interval (CI): 0.62 – 0.84). Internal validation gave an AUC–ROC for ARDS of 0.71 (CI: 0.47 – 0.95). The diagnostic accuracy for the complete training cohort can be found in table 2, together with the optimal sensitivity and specificity.
**Temporal external validation**

The eNose provided an ROC-AUC of 0.71 (CI: 0.54-0.87) in the temporal external validation cohort (table 2). 27 of the 1000 random permutation tests resulted in a higher AUC-ROC, which means that chances of false discovery are 2.7%. A similar diagnostic accuracy was obtained with external validation using another eNose of the same manufacturer (AUC-ROC of 0.73 (CI: 0.58 – 0.90)).

**Subgroup analyses**

The predicted probability of group membership by the eNose (result of logistic regression) was significantly different between moderate/severe ARDS, and mild ARDS (0.45 vs. 0.36, \( P = 0.01 \)). The discrimination between moderate or severe ARDS and controls resulted in an AUC–ROC of 0.80 (CI: 0.70-0.90) with an optimal sensitivity of 91% and a specificity of 62%.

The predicted probability of group membership was not different between patients with a pulmonary (pneumonia, aspiration, etc) and a non–pulmonary cause (sepsis, pancreatitis, etc) for ARDS (0.41 vs. 0.38, \( P = 0.82 \)).

**Competing diagnoses**

The eNose signal was different between patients with pneumonia and patients with CPE from patients with ARDS, but with borderline significance levels (\( P = 0.05 \) and \( P = 0.05 \) vs. ARDS, respectively). Statistical significance and discrimination increased when patients with CPE and pneumonia were compared to patients with moderate/severe ARDS (\( P = 0.003 \) and \( P = 0.01 \); table 2).

**Sensitivity analysis**

The influence of co–variates on the association between exhaled breath and ARDS was assessed by comparing the log odds–ratio of the signal derived from the eNose (4.9 (CI: 2.5 - 7.6)) for ARDS in an unadjusted logistic regression model to the log odds-ratio found in a logistic regression model adjusted for the co– variate (table 3).
## Table 1: Patient characteristics

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<tr>
<th></th>
<th>Control (n = 92)</th>
<th>ARDS (n = 58)</th>
<th>Pneumonia (n = 11)</th>
<th>CPE (n =19)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (years)</strong></td>
<td>64 (50–75)</td>
<td>57 (54–78)</td>
<td>56 (49–62)</td>
<td>71 (63–79)</td>
<td>0.106</td>
</tr>
<tr>
<td><strong>Male (yes)</strong></td>
<td>51 (55)</td>
<td>30 (52)</td>
<td>8 (89)</td>
<td>11 (73)</td>
<td>0.327</td>
</tr>
<tr>
<td><strong>APACHE II</strong></td>
<td>20 (15–26)</td>
<td>23 (19–29)</td>
<td>20 (16–24)</td>
<td>23 (20–28)</td>
<td>0.013</td>
</tr>
<tr>
<td><strong>SAPS II</strong></td>
<td>48 (37–60)</td>
<td>55 (43–67)</td>
<td>49 (37–55)</td>
<td>57 (46–63)</td>
<td>0.013</td>
</tr>
<tr>
<td><strong>Admission type</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medical</td>
<td>56 (62)</td>
<td>41 (72)</td>
<td>7 (64)</td>
<td>16 (89)</td>
<td></td>
</tr>
<tr>
<td>Elective surgery</td>
<td>5 (6)</td>
<td>2 (4)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0.373</td>
</tr>
<tr>
<td>Emergency surgery</td>
<td>29 (32)</td>
<td>14 (25)</td>
<td>2 (11)</td>
<td>4 (36)</td>
<td></td>
</tr>
<tr>
<td><strong>Asthma</strong></td>
<td>1 (1)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>1 (5)</td>
<td>0.606</td>
</tr>
<tr>
<td><strong>COPD</strong></td>
<td>8 (9)</td>
<td>6 (10)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0.773</td>
</tr>
<tr>
<td><strong>Other respiratory</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Malignancy</strong></td>
<td>5 (5)</td>
<td>2 (3)</td>
<td>1 (9)</td>
<td>1 (5)</td>
<td>0.290</td>
</tr>
<tr>
<td><strong>DM</strong></td>
<td>7 (7)</td>
<td>13 (22)</td>
<td>1 (9)</td>
<td>1 (5)</td>
<td>0.090</td>
</tr>
<tr>
<td><strong>Pmax (cmH₂O)</strong></td>
<td>16 (11–20)</td>
<td>21 (15–30)</td>
<td>16 (15–24)</td>
<td>24 (19–29)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td><strong>PEEP (cmH₂O)</strong></td>
<td>5 (5–6)</td>
<td>8 (5–10)</td>
<td>5 (5–8.5)</td>
<td>8 (5–10)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td><strong>Tidal volume (ml)</strong></td>
<td>456 (393–545)</td>
<td>426 (380–494)</td>
<td>482 (451–579)</td>
<td>410 (373–506)</td>
<td>0.345</td>
</tr>
<tr>
<td><strong>Minute volume (l/min)</strong></td>
<td>8.5 (7.4–9.1)</td>
<td>11.0 (9.1–13.3)</td>
<td>11.3 (9.8–12.6)</td>
<td>9.6 (7.9–12.1)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td><strong>PaCO₂ (kPa)</strong></td>
<td>5.1 (4.6–5.7)</td>
<td>5.4 (4.6–5.9)</td>
<td>4.7 (4.1–5.6)</td>
<td>4.8 (4.6–5.6)</td>
<td>0.452</td>
</tr>
<tr>
<td><strong>PaO₂/FiO₂ (mmHg/%)</strong></td>
<td>311 (234–398)</td>
<td>212 (165–257)</td>
<td>304 (241–447)</td>
<td>242 (176–264)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td><strong>Leucocytes 10⁶/ml</strong></td>
<td>12.9 (10.2–18.2)</td>
<td>13.4 (8.7–19.4)</td>
<td>14.1 (13.6–15.1)</td>
<td>18.0 (16.0–18.9)</td>
<td>0.053</td>
</tr>
<tr>
<td><strong>CRP mg/ml</strong></td>
<td>65 (21–129)</td>
<td>144 (74–237)</td>
<td>135 (62–177)</td>
<td>66 (18–110)</td>
<td>0.002</td>
</tr>
<tr>
<td><strong>ICU Mortality</strong></td>
<td>16 (18)</td>
<td>20 (35)</td>
<td>0 (0)</td>
<td>6 (33)</td>
<td>0.023</td>
</tr>
</tbody>
</table>
### Table 2: Diagnostic accuracy of electronic nose analysis

<table>
<thead>
<tr>
<th>Comparison</th>
<th>ROC-AUC</th>
<th>Specificity</th>
<th>Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Training</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ARDS vs. Control</td>
<td>0.72 (0.63-0.82)</td>
<td>42%</td>
<td>95%</td>
</tr>
<tr>
<td><strong>External validation</strong></td>
<td></td>
<td></td>
<td></td>
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<td>ARDS vs. Control</td>
<td>0.71 (0.54-0.87)</td>
<td>50%</td>
<td>89%</td>
</tr>
<tr>
<td><strong>In-set: Subgroup analysis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moderate/severe ARDS vs. Control</td>
<td>0.80 (0.70-0.90)</td>
<td>62%</td>
<td>91%</td>
</tr>
<tr>
<td>Mild ARDS vs. Control</td>
<td>0.71 (0.54-0.87)</td>
<td>50%</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CPE vs. ARDS</td>
<td>0.76 (0.61-0.92)</td>
<td>74%</td>
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</tr>
<tr>
<td>Pneumonia vs. ARDS</td>
<td>0.69 (0.49-0.88)</td>
<td>83%</td>
<td>89%</td>
</tr>
<tr>
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### Table 3: Sensitivity analysis for potential confounders

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<th>P-value for eNose signal</th>
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<tbody>
<tr>
<td>eNose unadjusted</td>
<td>4.9 (2.5-7.6)</td>
<td>0.0001</td>
</tr>
<tr>
<td>eNose + SAPSII</td>
<td>4.98</td>
<td>0.0001</td>
</tr>
<tr>
<td>eNose + APACHE II</td>
<td>3.67</td>
<td>0.0089</td>
</tr>
<tr>
<td>eNose + Minute volume</td>
<td>4.98</td>
<td>0.0002</td>
</tr>
<tr>
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<td>0.0002</td>
</tr>
<tr>
<td>eNose + Comorbidities</td>
<td>4.98</td>
<td>0.0001</td>
</tr>
<tr>
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</table>
Discussion

This study with a commercially available eNose suggests that breath analysis might be used to identify patients with ARDS if the eNose technology would mature towards this application with increased diagnostic accuracy and sensor stability. The diagnostic accuracy was good for moderate/severe ARDS. These findings were confirmed by temporal external validation. Notably, the exhaled breath profile from patients with CPE and pneumonia was well distinguished from that of patients with moderate/severe ARDS. These data support the suggestion that eNose assessment may qualify as a candidate test for future non-invasive diagnostic approaches of ARDS.

This is the first study to look at the diagnostic accuracy of an eNose for the diagnosis of ARDS. Earlier studies focussed on biological makers in broncho–alveolar lavage fluid or exhaled breath condensate [12, 20, 39-42]. These sampling methods are time–consuming and sample analysis is not available in the intensive care unit. A pioneer paper by Schubert et al. reported on gas–chromatography and mass–spectrometry of the exhaled breath in ARDS patients, thereby detecting specific compounds in the breath [13]. The concentration isoprene was reported to be significantly lower in the breath of ARDS patients; however, neither sensitivity nor specificity was given. The present data extend those results by providing the diagnostic accuracy of exhaled breath profiling.

The reported AUC-ROC of 0.71 provides moderate accuracy and is lower than previously found accuracies using the same type of eNose, when discriminating between other pulmonary diseases. For example, the externally validated diagnostic accuracy was 0.95 when discriminating between asthma and COPD [43]. Several explanations can be given. First, alterations of exhaled VOC patterns may not always occur during ARDS or do also occur in ICU patients without ARDS. Second, the index–test may not be sufficiently accurate, as may be suggested by sensors drift, even though we carefully dealt with that. Finally, the reference–test may not be perfect, which is not uncommon in diagnostic research [44].

The gold–standard is an inherent problem in current diagnostic research of ARDS. Indeed, the new ARDS definition was found to be 89% sensitive but only 63% specific for diffuse alveolar damage, the histological hallmark of ARDS [2, 45, 46]. This discordance was most profound in patients with
mild ARDS. In the present study, we were not able to obtain the histo-
pathological gold standard for ARDS. In general, lack of a gold–standard
attributes to a lower observed diagnostic accuracy [44]. In our study, we
found an increasing likelihood for correct classification with increasing
severity of ARDS. Furthermore, there was no difference in discrimination
between patients with a pulmonary and a non–pulmonary causal factor
for ARDS. These findings are in line with the hypothesis of an imperfect
reference standard and indirectly support the validity of exhaled breath
analysis for the diagnosis of ARDS.

Patients with ARDS were discriminated from patients pneumonia and CPE
with modest accuracy. However, differentiation between these disease
states is regarded as one of the major clinical challenges in this patient
population and in this scenario the eNose does not seem to provide answers.
Diagnostic accuracy did increase when only patients with moderate/severe
ARDS were regarded as cases, but was still moderate. Interestingly,
the discrimination between moderate/severe ARDS and controls was
profoundly sensitive whilst comparison to pneumonia was mostly specific.
Thus ARDS can be excluded with confidence when compared to control
subjects while it can’t be when compared to pneumonia patients. Possibly,
some patients in the pneumonia group actually had ARDS but chest x-ray
was too insensitive to detect the bilateral infiltrates. Alternatively, some
patients with ARDS also had pneumonia and the differences in exhaled
VOCs was just too small to separate these phenotypes adequately.

One of the strengths of this paper is the assessment of the external validity
of the diagnostic algorithm. External validation is strongly recommended
to limit false–discovery and over–fitting of diagnostic models [30]. Other
strong points of this study may be represented by the recruitment of a
relatively large number of patients, completely independent assessment of
both index (exhaled breath analysis) and reference–test (ARDS diagnosis)
and pre–defined subgroup analyses. Although the use of two ARDS
definitions may seem a possible limitation we feel that we handled this
carefully as all analyses were performed with the Berlin definition, which
is more clearly defined with regards to disease severity and radiological
criteria.

The implicit limitation of this study is that the VOCs altered in ARDS
were not identified. This would require the use of gas–chromatography
and mass–spectrometry (GC–MS), currently the best method for VOC–
detection [47]. This is certainly required for understanding of the underlying
pathophysiological pathways leading to altered VOC concentrations in
the exhaled breath. VOC–identification was beyond the objective of the
present study, because we aimed to establish diagnostic accuracy in
the clinical setting. To that end, we performed sensitivity and specificity
analysis based on composite VOC–signals, thereby taking maximal benefit
of the multiple (as yet unknown) biomarkers involved. Therefore, eNose
technology is adequate for testing hypotheses on diagnostic accuracy [25].
Diagnosis by eNose is rapid, cheap and easy to perform and therefore
closer to clinical applicability than most other methods for exhaled breath
analysis available at this moment.

Second, we cannot exclude that patient–related factors such as ventilation
strategies, therapy, comorbidities and exposure to metabolic active
compounds are (partly) responsible for the altered exhaled breath signal.
However, sensitivity analyses showed that ventilator settings such as
minute volume ventilation and comorbidity are probably not responsible
for the found signal. It is difficult, if not impossible, to control for all
confounders in an observational study but this can be accomplished in pre–
clinical experiments. Importantly, lipopolysacharide–induced lung injury
was found to induce changes in exhaled breath profiles in three separate
experimental rat models [11, 48]. It may very well be that a similar signal
was detected in the present study, using a different analytical technique.

The prevalence of ARDS was high in the studied patient cohort, and higher
than in most previous cohort studies. Several factors could serve as an
explanation for this discrepancy. First, included patients were severely ill,
as suggested by the high disease severity scores and the high mortality.
Second, different from other cohorts of critically ill patients, we excluded
patients after cardiopulmonary surgery. Finally, ARDS was assessed
prospectively by a team of trained research fellows. Prospective assessment
may identify patients that could have been missed retrospectively.

In this study, we used SPLS logistic regression analysis for model
development. This algorithm can be used for variable selection in high
dimensional datasets with low numbers of patients, while maintaining
external validity and limiting false discovery [38]. Another advantage of
SPLS is that the produced model is relatively simple to interpret. Some
sensors were found to be predictive of ARDS when the sensor result was lower compared to control, as indicated by a negative coefficient in the model (table 2). This is probably due to lower breath concentrations of VOCs with affinity to these sensors. Following GC–MS driven research, we can hypothesize that isoprene can be one of these VOCs [13]. Interestingly, not all coefficients were negative: apparently three sensors are affinitive for VOCs that increase in concentration. Combined, these findings provide evidence that ARDS is associated with both up–regulation and down–regulation of volatile metabolites.

This paper describes exhaled breath analysis as a diagnostic tool for ARDS. However, several steps need to be taken before exhaled breath analysis can be implemented into clinical practice. Primarily, we need a list of potential ARDS–biomarkers in exhaled air obtained from controlled pre–clinical models, thereby excluding confounders as medication, comorbidities and ventilatory strategies. Second, the accuracy of sensors needs to be increased as the tested commercially available technology proved insufficient: sensor sensitivity and specificity for VOCs can be modified targeting potential biomarkers. Drift should be minimized and sensor–arrays should provide interchangeable results to allow for application in large clinical trials. Continuous exhaled breath analysis would allow for monitoring. Finally, the lack of a gold standard cannot be solved easily. In the long run, a move from the diagnostic accuracy paradigm towards a test validation paradigm might be justified [44]. This would allow for the comparison of added value of several index–tests, including exhaled breath analysis, in clinical decision–making.

Conclusions

We found that an electronic nose can rapidly and non–invasively discriminate between patients with and without ARDS with modest accuracy. The diagnostic model was both externally validated and reproducible. Diagnostic accuracy increased when only moderate and severe ARDS patients were considered. The exhaled breath profile from patients with CPE and pneumonia was different from that of patients with moderate/severe ARDS.
Online supplement

For the online supplement, please use the following link, or scan the 2D code with your mobile phone.

www.biomedcentral.com/content/supplementary/1471-2466-14-72-s1.doc

References
Chapter 5.

Alterations of Exhaled Breath Metabolite–mixtures in Two Rat Models of Lipopolysaccharide–induced Lung Injury

Journal of Applied Physiology 2013

Abstract

Exhaled breath contains information on systemic and pulmonary metabolism, which may provide a monitoring tool for the development of lung injury. We aimed to determine the effect of an intravenous and an intra–tracheal lipopolysaccharide (LPS) challenge on the exhaled mixture of volatile metabolites and to assess the similarities between these two models.

Male adult Sprague–Dawley rats were anesthetized, tracheotomized and ventilated for six hours. Lung injury was induced by intravenous or intra–tracheal administration of LPS. Exhaled breath was monitored continuously using an electronic nose (eNose) and hourly using gas–chromatography and mass–spectrometry (GC–MS).

GC–MS analysis identified 34 and 14 potential biological markers for lung injury in the intravenous LPS model and the intra–tracheal LPS model, respectively. These volatile biomarkers could be used to discriminate between LPS challenged rats and control animals within 1 hour after LPS administration. eNose analysis resulted in a good separation 3 hours after the LPS challenge. Hexanal, 6,10–dimethyl–5,9–undecadien–2–one and pentadecane concentrations decreased after both intra–tracheal and intravenous LPS administration. Nonanoic acid was found in a higher concentration in exhaled breath after LPS inoculation into the trachea but in a lower concentration after intravenous infusion.

LPS–induced lung injury rapidly changes exhaled breath metabolite mixtures in two animal models of lung injury. Changes partly overlap between an intravenous and an intra–tracheal LPS challenge. This warrants testing the diagnostic accuracy of exhaled breath analysis for ARDS in clinical trials, possibly focusing on biological markers described in this study.
Introduction

The acute respiratory distress syndrome (ARDS) results either from an indirect insult (e.g., systemic inflammation with sepsis or pancreatitis), or from a direct pulmonary challenge (e.g., pneumonia, aspiration or inhalation trauma). Whatever its cause, ARDS is characterized by pulmonary inflammation, alveolar protein leakage and disrupted repair mechanisms [1]. The diagnosis of ARDS is based on clinical symptoms and radiological findings potentially hampering and delaying its recognition [2-5]. Biological markers may have the potential to improve and accelerate the diagnostic process of ARDS, but systemic biological markers (i.e., markers within blood) studied so far exhibit only limited diagnostic value [6, 7]. Therefore, recent efforts have focused on sampling local biological markers (i.e., markers within the pulmonary compartment) (7–9). This has demonstrated that multiple biological markers reflect ARDS better than one single biological marker [8, 9].

Extensive metabolic profiling (metabolomics) represents a comprehensive tool for biomarker discovery in complex metabolic mixtures [10]. Metabolomics of blood and broncho–alveolar lavage (BAL)–fluid shows changes in metabolism during pulmonary inflammation and injury [11-15]. Some of the produced metabolites are volatile and therefore detectable in the exhaled breath [16]. So–called volatile organic compounds (VOCs) can be separated, identified and quantified using gas–chromatography and mass–spectrometry (GC–MS), which is considered the gold standard for (volatile) metabolite discovery [17]. VOCs can also be rapidly detected on–site using sensor–based devices called electronic noses (eNose). This method does not allow for metabolite discovery, but provides probabilistic evidence for the presence or absence of disease by pattern recognition [18]. In other inflammatory diseases of the lung (e.g., asthma and chronic obstructive pulmonary disease) exhaled breath analysis has been examined for diagnostic purposes [19, 20]. Subsequently, several metabolites have been linked to cellular inflammation [21-24]. This suggests that volatile metabolic changes could also reflect inflammatory responses and injury with development of ARDS.

Ideally, potential biomarkers are investigated in high–risk patient groups using gold standard reference tests. However this is hampered by an
imperfect clinical reference standard, a high prevalence of co–morbidities and a veritable pharmacopeia of drugs in patients with ARDS [2]. Therefore, animal models of lung injury may allow for a first selection of potential early volatile biological markers of ARDS. Both systemic and local administration of lipopolysaccharide (LPS) lead to accumulation of inflammatory cells in the lung and disrupted barrier mechanisms similar to those found in the early phases of human ARDS and correspond to an indirect (e.g. sepsis) and direct (e.g. pneumonia) insults, respectively [25, 26].

We hypothesized that LPS–induced lung injury changes the mixture of exhaled metabolites, and that these changes overlap between systemic or local administration of LPS. To test these two hypotheses, we challenged rats with intravenous or intra–tracheal LPS in a model of ventilation [27].

Materials and methods

Animals

Adult specific pathogen–free Sprague–Dawley rats weighing ~ 400 grams (Harlan, the Hague, The Netherlands) were used in all experiments. The Animal Care And Use Committee of the Academic Medical Center at the University of Amsterdam, Amsterdam, The Netherlands, approved the study. All animal procedures were carried out in compliance with the Institutional Standards for Human Care and Use of Laboratory Animals.

Study design and experimental groups

This was a parallel study with 4 animal groups. Lung injury was induced by intravenous (IV) infusion or intra–tracheal (IT) instillation of LPS (7.5 mg/kg in 2 ml normal saline, or 1.0 mg/kg in 200 µl, respectively) (from Escherichia coli, L4131, Sigma Aldrich, Steinheim, Germany). Two control groups received an equal amount of sterile normal saline IV or IT. Each group consisted of 12 rats; per day, 2 rats were randomly assigned to one of the four groups and challenged.

Experimental protocol

Ventilation was performed as described before [27]. In short, anaesthesia was induced using a mixture of ketamine (Eurovet Animal Health B.V.,
Bladel, The Netherlands), dexmedetomidine (Pfizer Animal Health B.V., Capelle a/d IJsel, the Netherlands) and atropine (Pharmachemie, Haarlem, The Netherlands) and maintained with ketamine. The ventilation pattern could influence exhaled breath VOCs [28, 29]. Therefore, rats were tracheotomized and subsequently ventilated using a Servo 300 ventilator (Siemens, Upplands Väsby, Sweden) with tidal volumes of 6 ml/kg with positive end–expiratory pressure (PEEP) at 5 cmH₂O and a respiratory rate of 40/min or tidal volumes of 12 ml/kg with PEEP at 0 cmH₂O and a respiratory rate of 20/min. These two ventilation protocols represent the two extremes of clinically applied settings [30] and were used to investigate the robustness of the diagnostic algorithm with different ventilator settings. Tidal volumes were monitored using a pneumotachometer (HSE; Harvard Apparatus, Manheim, Germany) and adjusted if necessary. The lungs were recruited hourly and respiratory rate was adjusted based on hourly blood gas analysis (Rapidlab, Siemens, Erlangen, Germany) to keep normo–pH.

LPS was administered 30 minutes after the start of mechanical ventilation. Blood pressure and temperature were recorded continuously (Braun, Melsungen, Germany connected to Siemens SC900; Siemens Medical Systems Group, Danvers, Mass). If the mean arterial pressure fell below 65 cmHg, nor–adrenaline was started. Temperature was maintained between 36.5°C and 37.0°C using a warming pad (ama–digit ad 15th; Amarell, Kreuzwertheim, Germany). Urine was collected at baseline, and after 3 and 6 hours.

After 6 hours the rats were sacrificed. Lung wet–to–dry (WD) ratios were determined as described before [27], using the right lower lobe of the lung. To obtain BAL–fluid, 3 x 2 ml of normal saline was instilled in and directly withdrawn from the contra–lateral lung, and kept at 4°C until further processing.

**Measurements**

The number of cells in BAL–fluid was counted (Z2 Coulter Particle Counter; Beckman Coulter Corporation, Hialeah, Fla) and cell–differentiation with Giemsa Stain (Dade Behring AG, Dudingen, Switzerland) was performed. Total protein levels (Oz Biosciences, Marseille, France) and levels of tumor
necrosis factor (TNF)-α, interleukin (IL)-6, cytokine induced neutrophil chemoattractant (CINC)-3 and IL-10, (ELISA; R&D systems, Abingdon, United Kingdom) were measured in BAL-fluid.

Exhaled breath analysis

Measurements with eNose were performed in half the experimented animals. For this, directly after the initiation of ventilation, an eNose (Comon Invent, Delft, The Netherlands) was connected, via a T-piece, to the expiratory limb of the ventilator tubing. The eNose contains seven metal–oxide sensors, which reversibly bind a broad spectrum of VOCs. VOC-binding results in a change in electrical resistance, measured by altered electrical current, which was recorded continuously for 6 hours and stored every 10 seconds.

GC–MS analysis was performed in all experimented animals. For this, a new stainless steel tube containing Tenax GR (Tenax™ GR 60/80, Interscience, Breda, The Netherlands) was connected to the expiratory limb of the ventilatory circuit every hour. VOCs were absorbed onto this tube every hour for 30 minutes with a flow of 35 ml/minute. The Tenax tubes were transported to a thermal desorption unit (Markes TD100 Cincinnati, Ohio, USA) and heated to 280°C for 15 minutes with a flow of 30 ml/min. The VOCs were captured on a cold trap at 10°C, which was rapidly heated to 300°C for one minute, after which the molecules were split less injected through a transfer line at 180°C onto an Inertcap 5MS/Sil gas–chromatography column (30 m, ID 0.25 mm, film thickness 1 µm, 1,4–bis(dimethylsiloxy)phenylene dimethyl polysiloxane, Restek, Breda, The Netherlands) at 1.2 ml/min. The oven temperature was isothermal at 40°C for 5 minutes, then increased to 280 at 10°C/min and kept isothermal at 280°C for 5 minutes. Molecules were ionized using electron ionization (70 eV) and the fragment ions were detected using a quadrupole mass–spectrometer (GCMS–GP2010, Shimadzu, Den Bosch, the Netherlands) with a scan range of 37–300 Da. Ion–fragment peaks were used for statistical analysis. The predictive fragment–ions were manually checked in the raw chromatograms and the corresponding metabolites were tentatively identified based on NIST–library matching. Metabolites were considered identified if the first five hits in the library were the same compound and all matching factors were above 90%. In the case of multiple
likely library hits, a chemical standard (Sigma–Aldrich, Zwijndrecht, The Netherlands) was injected for identification. When these two procedures did not result in identification, the compound was called unidentified.

**Statistical Analysis**

Primarily, the groups challenged with LPS were compared with their corresponding controls. Secondarily, intravenous and intra–tracheal LPS–induced lung injury groups were compared using a Venn diagram [31]. Time dependent changes between groups in continuous variables were analyzed using linear mixed models. Differences in end points that were measured once were analyzed using the Mann–Whitney U test. P–values below 0.05 were considered significant. All analysis were performed in R–studio (V 0.97) using the statistical data environment R (V2.15) [32].

GC–MS analysis, de–noising, peak–detection and alignment were performed using the Xcms–package [33] (Scripps center for metabolomics, La Jolla, CA, USA) and resulted in an ion–fragment peak table as input for statistical analysis. eNose analysis resulted in seven sensor signals per 10 seconds, per animal. Fragmented–ion peaks and sensor signals were expressed as a percentage change of baseline measurement (first thirty minutes of mechanical ventilation, before LPS administration). Fragment–ion peaks were checked for outliers by principal component analysis. Outliers were defined as a chromatogram with many infinite values (compared to baseline) as detected by PCA. When no valid baseline chromatogram was available, all data from that animal was neglected.

Fragment–ion peaks that changed significantly after LPS administration were detected using six steps (figure 1). First, a linear mixed effect model was fitted using the peak as dependent variable with time after start of mechanical ventilation as independent variable and with a random intercept per animal. Time was modeled with a basic spline to allow for non–linear fluctuations in metabolites (e.g. increase in the first three hours after LPS and decrease thereafter). Secondly, this procedure was repeated with the addition of the LPS group (yes/no) as a second independent variable. Thirdly, the improvement of the model with the addition of LPS allocation was expressed as the difference in Akaike information criterion [34] (AIC) between the two models.
Figure 5.1: Selection of statistically significant ion-fragments.
Fourthly, step two and three were 1000 times repeated but with a random group allocation instead of LPS allocation to simulate chance findings. Fifthly, delta AICs were log–transformed and a normal distribution was fitted through the randomly generated values. Finally, the false–discovery rate (FDR) per AIC cut–off value was calculated and peaks with a FDR of below 5% were selected.

A logistic regression model was trained using the previously selected fragment–ion peaks. Sparse–partial least square (SPLS) logistic regression was used as an alternative to “normal” regression techniques in this study, because SPLS can limit false discovery in situations were a large number of independent variables are investigated in low numbers of individuals [35]. The mean and 95% confidence–interval (95%–CI) of the predicted probability of group membership over time was displayed in a figure.

A SPLS logistic regression model was also trained on the mean change in electrical resistance per sensor after 330 to 360 minutes of mechanical ventilation. This logistic regression model was there–after applied to all other sensor data.

## Results

### Animals

Of 55 included animals 8 rats died during instrumentation (figure 2). Forty–seven rats completed the experiment. Exhaled breath was continuously analyzed by eNose from 6 animals per group. Exhaled breath for GC–MS analysis was hourly obtained from all 47 rats. However, since baseline measurements were unsuccessful in four animals due to technical problems with GC-MS, GC–MS data from these animals were excluded from further analysis.

### Extra–pulmonary manifestations of LPS administration

Intravenous administration of LPS resulted in a drop in mean arterial blood pressure (p < 0.001, figure 3A), but the mean arterial blood pressure remained above 65 mmHg at all time points in all rats. These rats also had a lower pH throughout the experiment (p = 0.003, figure 3C), with the largest difference after 2 hours of start of ventilation. Urine output also
declined \((p = 0.007\), figure 3F\). Systemic blood pressure, pH and urine output were not affected by intra–tracheal administration of LPS (figure 3B, D, and F).

**Figure 2: Flowchart**

![Flowchart Diagram]

**Pulmonary manifestations of LPS administration**

Rats challenged with intravenous LPS had lower numbers of neutrophil cells in BAL–fluid \((p < 0.001\) vs. control\), while rats challenged with intra–tracheal LPS had higher numbers of neutrophil cells in BAL fluid \((p < 0.001\) vs. control\) (figure 4A, and B). All LPS–challenged rats showed signs of pulmonary inflammation, with higher levels of TNF–alpha \((IV\ p = 0.045\ vs.\ control;\ IT\ p < 0.001\ vs.\ control)\), IL–6 \((IV\ p < 0.001\ vs.\ control;\ IT\ p < 0.001\ vs.\ control)\) and CINC–3 in BAL fluids \((IV\ p < 0.001\ vs.\ control;\ IT\ p < 0.001\ vs.\ control)\) (figure 4C, and D).

**Alterations in exhaled metabolites analyzed by GC–MS**

372 and 355 mass–spectrometer generated fragmented ions of VOCs were present in all air samples from intravenous or intra–tracheal challenged rats, respectively. In the intravenous model, 120 ion–fragments were found to be significantly altered after LPS administration. In the intra–tracheal model, 31 ion–fragments were significantly altered after LPS inoculation.
Figure 3: Physiological changes after LPS infusion

Squares represent the median of the LPS group, round of the control group. The spread shows the minimum and maximum value per time point. MAP = mean arterial pressure.
Figure 4: Immune response after LPS administration

Discrimination between rats challenged with intravenous LPS and control animals was based on 30 ion–fragments. The logistic regression function diverged between the two groups 30 minutes after LPS administration or 60 minutes after the start of mechanical ventilation (figure 5A). The area under the receiver–operating characteristic curve (AUC–ROC) was 0.84 [95% CI: 0.66–1.0] 30 minutes after LPS administration and increased to 0.94 [95%–CI: 0.83–1.0] during the next hour. The AUC–ROC remained similar for the rest of the experiment.

Discrimination between rats challenged with intra–tracheal LPS animals and control animals was based on 13 ion–fragments. Discrimination was good (ROC–AUC: 0.94 [95%–CI: 0.84–1.0]) 30 minutes after LPS inoculation and remained good until the end of the experiment (figure 5B).
Alterations in exhaled breath profile analyzed by electronic nose

Continuous electronic nose analysis of the exhaled breath resulted in good discrimination between animals that were challenged with LPS intravenously and control animals after approximately 3 hours (ROC–AUC: 0.85 [95%–CI: 0.78–0.97]) (figure 6A). After intra–tracheal LPS administration, good discrimination was reached within 90 minutes (ROC–AUC: 0.82 [95%–CI: 0.52–0.93]) (figure 6B). Notably, the control group deviated from the baseline measurements instead of the LPS group.

Metabolite identification and model comparison

The 120 significant ion–fragments found in the intravenous LPS model corresponded to 35 metabolites, of which 21 could be tentatively identified (table 1). The intra–tracheal LPS challenge resulted in 31 significantly altered ion fragments that corresponded to 14 metabolites, of which 9 could be identified (table 2). 5 metabolites changed significantly after the challenge with LPS in both models, as shown in the Venn diagram (figure 7). Hexanal, 6,10–dimethyl–5,9–undecadien–2–one, pentadecane and an unidentified compound were found in lower concentrations after both pulmonary and intravenous LPS administration. In contrast, nonanoic acid was found in a higher concentration in the exhaled breath after LPS inoculation into the trachea but in a lower concentration after intravenous infusion.

Dependence upon ventilation protocol

The logistic regression algorithm was not dependent on the ventilation protocol that was used (p = 0.97 / p = 0.60, for intravenous or intra–tracheal LPS administration, respectively).
**Figure 5:** Predicted probability of LPS administration by GC-MS

Logistic regression function based on GC–MS detected ion–fragments. A – The blue line indicates the mean logistic function for intravenous LPS challenged animals, the grey line for the control group. Dashed lines show the 95% confidence interval. B – The yellow line indicates the mean logistic function for intra–tracheal LPS challenged animals, the grey line for the control group. Dashed lines show the 95% confidence interval.

**Figure 6:** Predicted probability of LPS administration by eNose

Logistic regression function based on electronic nose analysis. A – The blue line indicates the mean logistic function for intravenous LPS challenged animals, the grey line for the control group. Dashed lines show the 95% confidence interval. B – The yellow line indicates the mean logistic function for intra–tracheal LPS challenged animals, the grey line for the control group. Dashed lines show the 95% confidence interval.
<table>
<thead>
<tr>
<th>Name</th>
<th>M/Z</th>
<th>RT (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoflurane</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2,3-Butanedione</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-Butanone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trifluorobenzene (unknown isomer)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>163</td>
<td>310</td>
</tr>
<tr>
<td>Butanal, 3-methyl-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>38/49/79</td>
<td>367</td>
</tr>
<tr>
<td>Unknown</td>
<td>181</td>
<td>374</td>
</tr>
<tr>
<td>Hexanal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>82</td>
<td>683</td>
</tr>
<tr>
<td>3-Heptanone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclohexanone</td>
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<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>79/99/122</td>
<td>898</td>
</tr>
<tr>
<td>Hexanal, 2-ethyl-</td>
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<td></td>
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<tr>
<td>Unknown</td>
<td>101</td>
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</tr>
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<td>1-Hexanol, 2-ethyl-</td>
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<td></td>
</tr>
<tr>
<td>D-Limonene</td>
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<td>Unknown</td>
<td>50/51/62/89</td>
<td>1006</td>
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<tr>
<td>Benzenemethanol, dimethyl-</td>
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<td>145</td>
<td>1169</td>
</tr>
<tr>
<td>Unknown</td>
<td>94</td>
<td>1180</td>
</tr>
<tr>
<td>Nonanoic acid</td>
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<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>102/107/118</td>
<td>1206</td>
</tr>
<tr>
<td>Unknown</td>
<td>73</td>
<td>1241</td>
</tr>
<tr>
<td>n-Decanoic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>154</td>
<td>1338</td>
</tr>
<tr>
<td>5,9-Undecadien-2-one, 6,10-dimethyl-,</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-Dodecanol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pentadecane</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenol, 3,5-bis(1,1-dimethylethyl)-</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*RT: Retention time in seconds. M/Z: Mass over charge; the mass of the ion fragment(s)*
Table 2: Significantly altered metabolites in the intra–tracheal LPS model

<table>
<thead>
<tr>
<th>Name</th>
<th>M/Z</th>
<th>RT (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4–Hexadiyne</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Toluene</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hexanal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2–Cyclopenten–1–one</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclopentanone, 2–methyl–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2–Pentylfuran</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>56/86</td>
<td>902</td>
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<td>Unknown</td>
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<td>948</td>
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<tr>
<td>Unknown</td>
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<td>984</td>
</tr>
<tr>
<td>Unknown</td>
<td>95</td>
<td>995</td>
</tr>
<tr>
<td>Nonanoic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>154</td>
<td>1332</td>
</tr>
<tr>
<td>5,9–Undecadien–2–one, 6,10–dimethyl–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pentadecane</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

RT: Retention time in seconds. M/Z: Mass over charge; the mass of the ion fragment(s).

Figure 7: Venn diagram

Venn diagram for the two lung injury models, intravenous LPS (left) and intra–tracheal LPS (right). Were the circles overlap, the number of shared differences is indicated.
Discussion

This animal study shows that LPS induces a metabolic response that can be measured in exhaled breath. Longitudinal analysis revealed that the volatile metabolic changes in the exhaled air can be used to discriminate between rats challenged with LPS and control animals within one hour after LPS administration. This could be reproduced with a bedside electronic nose, although it took longer to reach good discrimination. Finally, this study provides evidence that changes in exhaled breath metabolites partly overlap between intravenous and intra–tracheal LPS administration. These data suggest that exhaled volatile metabolites may be used to monitor the development of ARDS.

To our knowledge, we performed the first experiment with longitudinal exhaled breath measurements in an animal model of acute lung injury, allowing for time–dependent analyses. The present data extend those by Gauman et al. [36], who observed a similar discriminatory power at a single time point, 24 hours after intra–peritoneal LPS–administration, and show that changes in exhaled VOCs already occur shortly after LPS instillation. However, the discriminating metabolites are different in both studies, which may be due to afore mentioned differences in study design.

Two complementary analytical techniques were used for the analysis of exhaled breath. GC–MS allowed for separation and identification of individual VOCs while eNose technology relies on pattern recognition. Arguably, pattern recognition is sufficient for clinical practice. This is the first study to perform continuous exhaled breath analysis during ventilation using eNose technology. The observed discrimination by eNose analysis in the intravenous LPS model mimics that of the GC–MS measurements, albeit later. However, after the intra–tracheal LPS challenge, the control group deviated from the baseline measurement instead of the challenged group. This suggests that another signal dominates or that the sensors in this eNose are not sensitive to the significantly altered volatile metabolites found by GC–MS but to unknown other compounds that apparently change after intra–tracheal saline administration.

The results of both challenge models were combined into a Venn diagram, which revealed that five metabolites were altered in both models. This
method has been proposed for meta–analysis in unbiased metabolomics
research and limits false–discovery through external validation [31].
Four of the five shared metabolites were found in lower concentration
after challenge with LPS in both models. Hexanal, is a breakdown
product of oxidized linoleic acid and therefore a marker of oxidative
stress [37]. Hexanal concentrations in exhaled air were previously found
to be increased in COPD and lung cancer patients [38, 39]. Exhaled air
concentrations of hexanal was found to be lower in smoking subjects,
when compared to controls and lung cancer patients [40]. Apparently,
exhaled hexanal is differentially associated with oxidative stress in health
and disease, which may be related to differences in local oxidative stress
and/or activation of alternative pathways. In line with these findings,
pentadecane (a classic marker of oxidative stress) was also found in
lower concentrations after LPS administration in our experiments. 6,10–
dimethyl–5,9–undecadien–2–one is a product of acetone termination
of geranyl phosphate, an intermediate in the sterol pathway [41]. We
speculate that the lower concentration of this metabolite in exhaled air
with development of lung injury could be explained in two ways. First, the
sterol pathway is down–regulated after LPS–administration. Second, the
specific shunt pathway through 6,10–dimethyl–5,9–undecadien–2–one
could be reduced in order to increase cholesterol and steroid hormone
synthesis. The latter is supported by studies showing that cholesterol
and steroid hormone concentrations increase after endotoxin infusion
(33, 34). The fourth shared marker was not identified. To summarize,
previously described exhaled markers of oxidative stress were found in
lower concentration after LPS administration. Possibly, however unlikely,
oxidative stress is decreased after LPS administration. Alternatively,
oxidative stress may cause activation of alternative pathways under
different circumstances. This is very much in line with exhaled NO, which
is a marker of oxidative stress when exhaled in either higher or lower
concentrations [42].

Interestingly, nonanoic acid was found in a higher concentration in the
exhaled breath of rats that received LPS in the trachea but in a lower
concentration when LPS was infused intravenously. Nonanoic acid is a
ligand to the Orphan G protein–coupled receptor GPR84 [43]. This receptor
is mainly expressed by leukocytes and induces an inflammatory response
(leukocyte influx and activation) when stimulated [43]. The present data are suggestive of a relationship between nonanoic acid concentration and the number of neutrophils in the broncho–alveolar lavage fluid, as these follow similar trends between the two models (lower after intravenous LPS and higher after intra–tracheal LPS) (figure 4A–B).

Our study knows several limitations. Some of the significant metabolites could not be identified because the mass–spectrum was not sufficiently informative. In the future, this could be overcome by applying high resolution mass–spectrometry or the inclusion of more external standards of known metabolites. Additionally, very low molecular weight volatile organic compounds (C1–C3) could not be monitored due to their limited retention on the absorbent tubes used in these experiments. Future studies aiming at these compounds should use a multi–sorbent VOC trap or new, rapid analytical techniques that do not require sample transportation (e.g. ion–mobility spectrometry [36, 44] or proton–transfer mass–spectrometry [45]).

A second weakness of this study is the limited number of animals investigated. Unfortunately, small numbers per group and a highly dimensional dataset result in increased chances of type I errors [46]. However, we limited false–discovery by repeating the statistical procedure with random group allocation and selection of only those peaks with a FDR of below 5%. Furthermore, true external validation was applied through the earlier described process of meta–analysis, thereby further reducing the chances on false–discovery for hexanal, pentadecane 6,10–dimethyl–5,9–undecadien–2–one and an unidentified fourth metabolite as biomarkers for lung injury. On the other hand, this stringent procedure inevitably promotes false negative results. Hence, our data do not exclude that other described markers can be representative for the phenotypic presentation of lung injury due to a pulmonary or a non–pulmonary challenge.

The mild nature of the used LPS–models could be considered a final limitation. Indeed, larger differences between the groups would probably have been found if a higher dose of LPS, injurious mechanical ventilation or a combination of both was used. However, since several physiological parameters (e.g., alveolar ventilation, cardiac output, ventilation/
perfusion mismatch) influence the concentration of VOCs in the exhaled breath, the investigated model should allow for maintaining the physiology within normal levels [47]. In our experiments, we were able to rapidly counteract disturbances in homeostasis due to LPS, because the rats were instrumented and monitored continuously before the administration of LPS. This required multiple interventions: shock was prevented by fluid and noradrenalin infusion, acid–base imbalance by bicarbonate infusion and changes in respiratory rate and atelectasis by hourly recruitment maneuvers. These are all risk factors for ARDS [48]. Hence, by keeping LPS-induced injury relatively mild, we aimed to limit the confounding effects of gross physiological disturbances. However, the given dosage was sufficient to induce an inflammatory response comparable to that observed in humans. Finally, an early marker for lung injury is clinically required. Waiting for a longer period (e.g. 24 hours) after LPS administration, as in other models, was therefore not desirable.

The present data suggests that exhaled breath metabolites may be used to monitor lung injury. If these results can be translated to the clinical setting, this would provide intensive care physicians with a non-invasive diagnostic tool that allows for continuous monitoring.

To conclude, LPS–induced lung injury rapidly changes the mixture of exhaled metabolites in two animal models. Alterations in exhaled breath metabolites can be detected as soon as one hour after LPS administration. These results were partly reproduced by exhaled breath profiling with an electronic nose. Exhaled breath metabolite alterations partly overlap between rats challenged with LPS intravenously and intra–tracheally. We identified the following biomarkers for lung injury in general: hexanal, pentadecane 6,10–dimethyl–5,9–undecadien–2–one and an unidentified fourth metabolite. Furthermore, exhaled nonanoic acid could be a marker for alveolar neutrophil influx. We also described 30 potential volatile biomarkers for non–pulmonary lung injury and 9 potential volatile biomarkers for pulmonary lung injury. This implies that diagnostic accuracy of exhaled breath analysis for ARDS should now be further investigated in clinical trials, possibly focusing on the biomarkers described in this pre–clinical study.
References


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Chapter 6.

Exhaled breath metabolomics as a non-invasive diagnostic tool for ARDS

European Respiratory Journal 2014

Lieuwe DJ Bos, Hans Weda, Yuanyue Wang, Hugo H Knobel, Tamara ME Nijsen, Teunis J Vink, Aeilko H. Zwinderman, Peter J Sterk & Marcus J Schultz
Abstract

There is a need for biological markers in the acute respiratory distress syndrome (ARDS). Exhaled breath contains hundreds of metabolites in gas phase, some of which reflect (patho-)physiological processes. We aimed to determine the diagnostic accuracy of metabolites in the exhaled breath for ARDS.

Breath from ventilated ICU-patients (n=101) was analyzed using gas-chromatography and mass-spectrometry during the first day of admission. ARDS was defined by the Berlin definition. Training and a temporal validation cohort were used.

23 patients in the training cohort (n=53) had ARDS. Three breath metabolites, octane, acetaldehyde and 3-methylheptane, could discriminate between ARDS and controls with an area under the receiver operating characteristics curve (ROC-AUC) of 0.80. Temporal external validation (19 ARDS, n=48) resulted in a ROC-AUC of 0.78. Discrimination was insensitive to adjustment for severity of disease, a direct or indirect cause for ARDS, comorbidities or ventilator settings. Combination with the lung injury prediction score increased the ROC-AUC to 0.91 and improved net reclassification by 1.17.

Exhaled breath analysis showed good diagnostic accuracy for ARDS, which was externally validated. These data suggest that exhaled breath analysis qualifies for the diagnostic assessment of ARDS.
Introduction

As stated in the updated and improved consensus criteria for acute respiratory distress syndrome (ARDS), a valid and reliable definition for ARDS is considered essential for clinical management and to facilitate enrolment of consistent patient phenotypes into clinical trials [1]. The presently used Berlin criteria are empirically selected clinical, radiological and physiological variables [1]. This definition is highly suitable for epidemiological studies but show a moderate correlation with post-mortem pathological findings [2]. ARDS can still be mistaken for pneumonia or cardiogenic pulmonary edema (CPE), and vice versa [2, 3]. Therefore there is need for molecular markers to group phenotypes more objectively and consistently [4].

A diagnostic molecular marker should improve classification on top of the pre-test probability of disease [5, 6]. In the case of ARDS, risk factors and risk modifiers have been thoroughly assessed, externally validated and incorporated into the Lung Injury Prediction Score (LIPS) [7, 8]. Discovery and validation of biological markers that reflect the pathophysiological mechanisms underlying lung injury may allow for improved diagnosis before the clinical definition of ARDS is met [9].

Metabolomics can provide an integrated view of upstream physiological, genomic, transcriptomic and proteomic data by assessment of the composition of metabolite mixtures in biological material [10-12]. Exhaled breath contains metabolites that are volatile [13]. Importantly, opposed to exhaled breath condensate collection, analysis of volatile organic compounds (VOCs) in gas phase does not rely on analysis of soluble markers. Instead, volatile metabolites can be trapped onto a sorbent tube and detected using gas–chromatography and mass–spectrometry (GC–MS) [14, 15]. Exhaled VOCs can be of systemic origin, can be produced in the lung (e.g. under influence of oxidative stress and inflammatory response) or can be the result of bacterial metabolism [16-21]. Pre–clinical studies provide evidence that lung injury induces changes in exhaled metabolites [17, 22]. Therefore, it is timely to validate the diagnostic accuracy of exhaled metabolomics in ARDS, by using a training set as well as a validation set of patients and controls [23].
We hypothesized that exhaled breath analysis by GC–MS can accurately diagnose ARDS in ventilated ICU-patients. We aimed to investigate the accuracy, reproducibility and robustness of this diagnosis and the classification of patients with CPE and pneumonia using exhaled breath analysis. This was done by following international guidelines on validating diagnostic accuracy as provided by STARD [24] (Table E1). Finally, we studied the classification performance for ARDS of exhaled breath analysis when combined with the a–priori risk, represented by the LIPS.

**Methods**

*Ethical approval and informed consent*

The institutional review board of the Academic Medical Center, Amsterdam, The Netherlands, decided that the study did not fulfil the criteria for medical research as stated in the Dutch ‘Law on medical research’ because of the non-invasiveness and absence of burden of examining exhaled air (IRB: 10.17.0729). It was judged by the institutional review board that exhaled breath should be analyzed without informed consent of the patient. This trial was registered at the Dutch Trial Register (NTR 2750, www.trialregister.nl).

*Design, subjects and setting*

This was a prospective single centre cohort study. All patients admitted to the ICU, with the exception of cardiopulmonary surgery patients, were screened between December 2011 and April 2013. The only inclusion criterion was mechanical ventilation within the first 24 hours of ICU-admission. The only exclusion criteria were previous ICU admission or mechanical ventilation. Patient who were included before June 2012 entered the training cohort and the remaining patients were used for temporal external validation [25]. Patients were categorized into four groups: controls, ARDS, pneumonia without ARDS and CPE.

*Clinical diagnosis of ARDS*

A team of trained clinical research fellows prospectively scored the presence of ARDS [26], which was later re–evaluated according to the new Berlin definition [1]. All observers were trained on several occasions.
before the start of the study. All assessors had attended meetings in which clinical case vignettes were discussed and had at least 6 months of work experience [27].

**Competing diagnoses**

The diagnosis of community- or hospital-acquired pneumonia consisted of adapted Center for Disease Control–criteria and a post–hoc likelihood of infection was scored (none, possible, probable or proven; see figure E1 and table E2 in the *supplementary material*) [27, 28]. In contrast to ARDS, the diagnosis of CPE required that the findings (acute onset, bilateral infiltrates and PaO2/FiO2 ratio < 300) were fully explained by cardiac dysfunction based on echocardiography [1].

**Exhaled breath analysis**

Exhaled breath was sampled and analyzed by standardized methodology that was previously published [29]. In short, breath was collected through a disposable side-stream connection for 10 minutes and VOCs were stored on a sorbent tube. These tubes were analyzed by means of GC-MS.

**Statistical analysis**

Differences between the groups were compared using the Mann–Whitney U or Kruskal–Wallis test for continuous variables and chi–square for categorical variables. Data was summarized using the median and 25–75th percentile for skewed variables and with mean and 95%–confidence interval (CI) for normally distributed variables and with count and percentage for categorical variables. All analyses were performed in R statistics using the R–studio interface [30]. P–values below 0.05 were considered significant.

The metabolomics data were analysed using an a priori data analysis plan (figure 1) that followed the latest recommendations for metabolomics [31, 32] and is described per step here and in the results section. The first step was **feature selection and training of algorithm**. To select the most relevant features, the training cohort was repeatedly split into a 2/3 training set and a 1/3 test set. 1000 iterations of sparse–partial least square (SPLS) logistic regression analysis (K=3, eta=0.9) were performed. This is a
method for penalized feature selection and is a form of regression that can select predictive variables and limit false discovery in situations where large number of independent variables are investigated in low numbers of individuals [33]. The five most frequently chosen ion-fragments (number of co-variates in a model is number of included patients divided by 10 [34]) were selected. A logistic regression model was fitted using the selected ion-fragments in the training cohort. We will refer to the result of that model as the “exhaled breath signal”. The logistic regression model was applied to the complete training cohort and the area under the receiver operating characteristic curve (ROC–AUC), with bootstrapped 95% confidence interval (95%–CI) was reported. The second and third step were to test the reproducibility and the temporal external validation by applying the logistic regression model to another breath sample, obtained at the same time point in the patients of the training cohort and to a new group of patients, respectively, and compare the ROC–AUC. The calibration of the predicted and observed probability of group membership was visualized in a correlation plot and displayed as figure E4 in the supplementary material. Step four was to perform a subgroup analyses for pulmonary/non–pulmonary and mild/moderate/severe ARDS. Step five was to investigate the diagnostic accuracy of the exhaled breath signal for competing diagnoses. Step six was a sensitivity analysis for comorbidities (chronic respiratory disease, malignancies and diabetes mellitus), bacterial growth in respiratory samples, ventilator settings and measures of severity of disease (Acute Physiology and Chronic Health Evaluation (APACHE) II and Simplified Acute Physiology Score (SAPS) II). Finally, the net reclassification improvement [6] of the combination with LIPS improves classification was investigated.

False-discovery and over-fitting was avoided by using a temporal external validation cohort [31, 32], with a required sample size of 18 ARDS patients to find 90% sensitivity with a lower confidence limit of 0.55 (i.e. statistical significance) [35].
**Figure 1:** Statistical analysis plan

Data after pre-processing

<table>
<thead>
<tr>
<th>#</th>
<th>Group</th>
<th>Ion-fragments (Abundance)</th>
<th>Matrix of abundances</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>ARDS</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Training 2/3

1. Selection of peaks
   SPLS

Model Fitting
   Logistic regression

2. Int. Validity
   Apply to Training set

3. Reproducibility
   Apply to other Tenax

4. Ext. Validity
   Apply Validation set

5. "Exhaled breath signal"
   Result logistic regression

**Figure 2:** Patient inclusion

140 Not eligible

99 Excluded

42 MV before

9 Logistics

1 Objection

7 Missed

160 Eligible

Training
53 Included

Validation
48 Included

53 Included
25 Control
23 ARDS
3 Pneumonia
2 CPE

48 Included
27 Control
19 ARDS
0 Pneumonia
2 CPE

Top: Inclusion chart

Bottom: Plan for statistical analysis, $\beta =$ regression coefficient per selected ion-fragment, SPLS = Sparse partial least square.
### Table 1: Patient characteristics

<table>
<thead>
<tr>
<th></th>
<th>Control (52)</th>
<th>ARDS (42)</th>
<th>CPE (4)</th>
<th>Pneumonia (3)</th>
<th>ADs (42)</th>
<th>Control (52)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (years)</strong></td>
<td>(60–44–70)</td>
<td>(67–48–75)</td>
<td>(57–39–68)</td>
<td>(69–66–71)</td>
<td>0.42</td>
<td>0.42</td>
</tr>
<tr>
<td><strong>Male (yes)</strong></td>
<td>(31 (70))</td>
<td>(35 (67))</td>
<td>(1 (33))</td>
<td>(1 (25))</td>
<td>0.16</td>
<td>0.16</td>
</tr>
<tr>
<td><strong>APACHE II</strong></td>
<td>(20–14–26)</td>
<td>(23–18–29)</td>
<td>(28–19–31)</td>
<td>(26–24–30)</td>
<td>0.12</td>
<td>0.12</td>
</tr>
<tr>
<td><strong>SAPS II</strong></td>
<td>(44–35–64)</td>
<td>(46–39–62)</td>
<td>(55–41–66)</td>
<td>(63–49–74)</td>
<td>0.079</td>
<td>0.079</td>
</tr>
<tr>
<td><strong>Admission type</strong></td>
<td>(34 (65))</td>
<td>(31 (74))</td>
<td>(2 (67))</td>
<td>(2 (50))</td>
<td>0.89</td>
<td>0.89</td>
</tr>
<tr>
<td></td>
<td>(10 (19))</td>
<td>(8 (19))</td>
<td>(1 (33))</td>
<td>(2 (50))</td>
<td>0.09</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>(3 (6))</td>
<td>(0 (0))</td>
<td>(0 (0))</td>
<td>(0 (0))</td>
<td>0.68</td>
<td>0.68</td>
</tr>
<tr>
<td></td>
<td>(4 (8))</td>
<td>(7 (16))</td>
<td>(1 (33))</td>
<td>(2 (50))</td>
<td>0.09</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>(6 (12))</td>
<td>(9 (20))</td>
<td>(0 (0))</td>
<td>(0 (0))</td>
<td>0.34</td>
<td>0.34</td>
</tr>
<tr>
<td><strong>Pmax</strong> (cmH(_2)O)</td>
<td>(16 (13–18))</td>
<td>(23.5 (18–29))</td>
<td>(15 (13–19))</td>
<td>(20 (18–22))</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Pplateau</strong> (cmH(_2)O)</td>
<td>(14.0 (12–18))</td>
<td>(22.5 (18–28))</td>
<td>(14.0 (12–18))</td>
<td>(18.5 (17–21))</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>PEEP</strong> (cmH(_2)O)</td>
<td>(5 (5–5))</td>
<td>(10 (5–10))</td>
<td>(5 (5–5))</td>
<td>(7.5 (5–10))</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Tidal volume</strong></td>
<td>(450 (391–510))</td>
<td>(486 (378–540))</td>
<td>(486 (397–510))</td>
<td>(490 (347–569))</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Vt/IBW</strong> (ml/kg)</td>
<td>(6.5 (5.6–7.5))</td>
<td>(6.8 (6.1–7.6))</td>
<td>(11.6 (9.7–14.6))</td>
<td>(7.3 (7.0–8.3))</td>
<td>0.057</td>
<td>0.057</td>
</tr>
<tr>
<td><strong>Minute volume</strong></td>
<td>(8.9 (7.5–10.9))</td>
<td>(10.9 (8.7–13.6))</td>
<td>(6.0 (6.0–7.6))</td>
<td>(7.2 (6.0–11.9))</td>
<td>0.014</td>
<td>0.014</td>
</tr>
<tr>
<td><strong>PaCO(_2)</strong> (kPa)</td>
<td>(5.1 (4.5–5.5))</td>
<td>(5.4 (4.7–6.0))</td>
<td>(5.1 (4.7–8.0))</td>
<td>(5.7 (5.0–6.0))</td>
<td>0.15</td>
<td>0.15</td>
</tr>
<tr>
<td><strong>PaO(_2)</strong> (kPa)</td>
<td>(14.7 (13.0–22.2))</td>
<td>(12.7 (11.0–15.5))</td>
<td>(15.7 (15.2–18.9))</td>
<td>(13.7 (13.4–15.0))</td>
<td>0.019</td>
<td>0.019</td>
</tr>
<tr>
<td><strong>FiO(_2)</strong> (%)</td>
<td>(35 (30–40))</td>
<td>(40 (40–56))</td>
<td>(40 (35–45))</td>
<td>(40 (39–43))</td>
<td>0.001</td>
<td>0.001</td>
</tr>
<tr>
<td><strong>PaO(_2)/FiO(_2)</strong> (mmHg/%)</td>
<td>(360 (281–454))</td>
<td>(357 (110–222))</td>
<td>(360 (110–222))</td>
<td>(357 (110–222))</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>LIPS</strong> (mmHg%)</td>
<td>(2.5 (1.5–4.0))</td>
<td>(5.0 (3.5–5.0))</td>
<td>(5.0 (3.5–5.0))</td>
<td>(5.0 (3.5–5.0))</td>
<td>0.014</td>
<td>0.014</td>
</tr>
</tbody>
</table>

*Table 1 (continued): Continuous variables are expressed as median (25th to 75th percentile). Categorical variables are expressed as number (percentage).*
Results

Subjects

Three hundred patients were screened, of whom 140 were not eligible and 59 met exclusion criteria (see figure 2). Thus, 101 patients were included. No adverse events were reported during or shortly after breath collection. Forty-two (42%) patients fulfilled the clinical definition for ARDS [1], 29 patients were classified as having mild ARDS, and 13 and 2 patients as moderate and severe ARDS, respectively (Table 1). 30 patients had ARDS due to a pulmonary cause (pneumonia (n=22), aspiration (n=2), other (n=6)) and 12 due to a non-pulmonary cause (sepsis (n=8), pancreatitis (n=1), other (n=3)). 52 (52%) patients did not fulfil the definition for ARDS; these patients served as control patients. Competing diagnoses were pneumonia (3 patients) and CPE (4 patients). None of the control patients progressed towards ARDS during the first three days of ICU-admission.

Feature selection and training of algorithm

Twenty-three patients with ARDS and 25 controls were included in the training cohort (Table E4 in the supplementary material shows baseline characteristics for this cohort). Five ion-fragments generated by the mass-spectrometer, which originated to three VOCs: octane (Chemical Abstract Service (CAS) registry number: 111–65–9), acetaldehyde (CAS: 75–07–0) and 3-methylheptane (CAS: 589–81–1), were selected by the algorithm (figure 1 and supplementary material) and were used as predictive variables in a logistic regression model (Table E3). Figure E2 and E3 in the supplementary material show the relative abundance of the fragments between patients with and without ARDS in the training cohort. The diagnostic accuracy of this exhaled breath signal for ARDS in the training-cohort was good (ROC–AUC: 0.80 [95%–CI: 0.66–0.92]; figure 3, dashed line).

Reproducibility

The exhaled breath signal derived from the 3 VOCs was reproducible using another breath sample taken from the same patient population within 10 minutes from the other sample (ROC–AUC: 0.78 [95%–CI: 0.65–0.91]; figure 3, dotted line).
Temporal external validation

19 patients with ARDS and 27 controls were included in the validation cohort (Table E5 in the supplementary material shows baseline characteristics for this cohort). The exhaled breath signal from the same 3 VOCs showed moderate to good diagnostic accuracy in a new cohort of patients (AUC–ROC: 0.78 [95%–CI: 0.65–0.91]; figure 3, solid line). Figure E4 in the supplementary material shows the relative abundance of the 5 fragments selected by the algorithm between patients with and without ARDS in the temporal external validation cohort.

Subgroup analyses

The logistic regression function was not different between patients with ARDS due to a pulmonary and a non–pulmonary hit (p = 0.24, figure E6 in the supplementary material). Discrimination of both pulmonary and non–pulmonary ARDS from controls was also not different (ROC–AUC: 0.75 [95%–CI: 0.63–0.86] and 0.80 [95%–CI: 0.63–0.96] for pulmonary and non–pulmonary ARDS, respectively, test between ROC–AUCs: p = 0.63 [36]).

The exhaled breath signal was not different between patients with mild or moderate/severe ARDS (p = 0.21, figure E6 in the supplementary material) and was not correlated with the PaO\textsubscript{2}/FiO\textsubscript{2} (Spearman’s correlation: 0.18, p = 0.27). Discrimination between ARDS and controls was also not different (test between ROC–AUCs: p = 0.19) between mild (ROC–AUC: 0.80 [95%–CI: 0.70–0.90]) and for moderate/severe ARDS (ROC–AUC: 0.65 [95%–CI: 0.44–0.86]).

Competing diagnoses

Discrimination of cardiopulmonary edema and pneumonia patients (not having ARDS) from ARDS was excellent (ROC–AUC: 0.91 [95%–CI: 0.80–1.0] and 0.90 [95%–CI: 0.81–1.0], for CPE and pneumonia, respectively).
Figure 3: Discrimination by breath analysis

Left: The diagnostic accuracy of breath analysis for ARDS was similar in the training and temporal external validation cohort. The dashed line shows the ROC-curve for the training cohort, the dotted line for reproducibility and the solid line for the validation cohort.

Figure 4: Breath analysis and LIPS

Right: The addition of breath analysis to the lung injury prediction score improves classification. The dashed line shows the ROC-curve for exhaled breath analysis, the dotted line for LIPS and the solid line for the combination.
Sensitivity analysis

The influence of co–variates on the association between exhaled breath and ARDS was assessed by comparing the log odds–ratio of the signal derived from the 3 VOCs (4.5 [95%–CI: 2.6–6.6]) for ARDS in an unadjusted logistic regression model to the log odds-ratio found in a logistic regression model adjusted for the co–variate. The log odds–ratio was not sensitive to changes in comorbidities (4.7), bacterial growth in respiratory samples (4.5), maximal inspiratory pressure (4.8), positive end–expiratory pressure (4.9), inspired oxygen fraction (4.6), ratio of pulmonary arterial oxygen tension to inspired oxygen fraction (5.7), smoking (4.7), alcohol consumption (4.7), APACHE II–score (4.3) and SAPS–II score (4.2).

Combination with LIPS improves classification

The LIPS score alone showed moderate/good discrimination between ARDS and controls (ROC–AUC: 0.78 [95%–CI: 0.70–0.87]) in the combined training and validation cohort. Discrimination improved when combined with the exhaled breath signal (ROC–AUC: 0.91 [95%–CI: 0.85–0.97], p=0.001 vs. LIPS alone, p=0.001 vs. exhaled breath signal alone; figure 4). The net reclassification improvement was 1.17 [95%–CI: 0.79–1.54] (p<0.001).

Discussion

This study shows that exhaled breath analysis provides good diagnostic accuracy for ARDS in ventilated ICU–patients. Discrimination was reproducible and the diagnostic performance was similar after temporal external validation. The exhaled breath diagnosis of ARDS was not influenced by severity of illness, ventilator settings or the examined comorbidities. Furthermore, the diagnostic accuracy increased when the exhaled breath analysis was combined with the LIPS. These findings indicate that exhaled breath analysis qualifies as diagnostic tool for the assessment of ARDS. Clinical application of this technology will be facilitated by further elevation of the diagnostic accuracy.

The described ROC–AUC of 0.80 for distinguishing ARDS patients from other ventilated ICU–patients is higher than most previously described
biological markers, such as interleukins, surfactant proteins and selectins [37]. In contrast to most other studies, we followed an untargeted approach for biomarker discovery. Our data extend previous ‘omics’ approaches using plasma or broncho-alveolar lavage (BAL) in diagnosing ARDS. A recent study that focused on metabolomics reported that the metabolite profile is altered in plasma of patients with sepsis-induced lung injury [38]. Previously, we argued that in the case of ARDS, preferably the lung should be assessed [15]. Indeed, experimentally induced lung injury promotes profound metabolomic changes in BAL and lung tissue [39, 40]. Taken together, our findings demonstrate that metabolomic profiling in ARDS is not only effective in plasma and BAL, but that exhaled breath also provides accurate diagnostic information on the altered metabolites in ARDS.

We identified three volatile biological markers of ARDS: octane, acetaldehyde and 3-methylheptane. However, we observed no differences in exhaled isoprene concentrations between patients with and without ARDS (figure E7 in the supplementary material), as reported in a pioneer paper by Schubert et al.[19]. The two studies differ on two major points: the conceptual approach and timing of patient selection. Concerning the first, we did not a-priori specify what compounds may be associated with ARDS, whereas the previous study was hypothesis-driven by focussing on exhaled acetone, isoprene and pentane. Second, our patients were included within 24 hours after ICU-admission, thereby early in the development of ARDS, whereas the patients in the previous study were included later during the course of disease. At the time, isoprene was linked to cholesterol metabolism and neutrophil activity but recent evidence connects isoprene to muscle activity [41]. In ICU-patients, muscle activity is affected by ICU-acquired weakness, coma, sedation and delirium. Thus, we could not reproduce the findings of the only previously published study on breath biomarkers for ARDS, which may be due to methodological differences or due to imbalances in confounding variables between the two groups in that study.

ARDS was associated with higher concentrations of octane in the exhaled breath. This VOC contributed most strongly to the diagnostic model. Octane is an end product of lipid-peroxidation, one of the degenerative processes caused by oxidative stress [42, 43]. Oxygen tension is known
to influence production of other hydrocarbons (e.g., pentane) but this did not apply to octane in our study as the sensitivity analysis showed that the log-odds ratio of the exhaled breath signal did not change when corrected for the inspired oxygen fraction. This may be explained by a difference in substrate of the peroxidation process; pentane is the result of peroxidation of n-6 PUFA, linoleic acid or arachidonic acid, while the proposed origin of octane is oleic acid [44]. The latter may not be as dependent on oxygen tension as the former, as suggested by our results. Of note, octane is also present in the breath of healthy individuals, but at very low concentrations [45]. and increases due to smoking [46], but this did not influence the diagnostic accuracy in our study.

We also found that higher concentrations of acetaldehyde and 3-methylheptane in the exhaled breath were predictive for ARDS. A variety of bacterial strains can produce acetaldehyde *in vitro* [16]. Bacterial colonization of the lung occurs frequently in ventilated ICU patients and this could have explained the association between ARDS and acetaldehyde. However, the findings that the sensitivity analysis showed no effects of airway colonization on the log–odds ratio for the exhaled breath signal and that patients with unilateral pneumonia included in this study were classified as not having ARDS suggest that bacterial metabolism is not a likely cause for the increased acetaldehyde concentration observed in patients with ARDS in this cohort. Acetaldehyde is also a product of leukocytes [47], and as neutrophil infiltration is a hallmark of ARDS [48] this may be a more appropriate explanation of the difference between patients with ARDS and patients without ARDS. Branched hydrocarbons like 3-methylheptane were suspected to be produced through lipid–peroxidation, similar to octane. However, alkenes and alkynes cannot result from lipid–peroxidation because there are no branched polyunsaturated fatty acids in the body [44]. To our knowledge, there have not been any reports on an alternative pathway for branched hydrocarbon production despite their association with many oxidative diseases, such as lung cancer [49]. This finding illustrates the hypothesis–generating aspect of metabolomics and deserves exploration. By and large, the exhaled breath signal appears to be mainly influenced by (pulmonary) oxidative stress and is further balanced by reflection of inflammatory or infectious processes and an as yet unidentified pathway. Their strength in assessing
ARDS appears to be their combination, which should be reflective of their partly independent metabolic pathways.

Our findings show that exhaled breath analysis provides information on the presence or absence of ARDS and this is complementary to pre-test risk assessment by the LIPS. This may not be unexpected as the LIPS consists of risk factors, whereas exhaled breath analysis is based on biochemical alterations that occur during ARDS. Hence, a patient can have a very high a-priori risk for disease (LIPS), but in absence of the biochemical presentation we may still conclude the patient probably does not have the condition (yet). In line with that, if a patient exhibits the biochemical profile with a negligible risk for disease such patient probably does not have the condition either. Our data provide the evidence that the combination of exhaled breath analysis with the LIPS can be highly suitable for identification of patients with ARDS.

Our study may have a number of strengths. First, the complex mixture of exhaled breath was profiled and the features most strongly linked to ARDS were selected, following the latest recommendations for metabolomics [31, 32]. The diagnostic accuracy of these biological markers was externally validated in a newly recruited population. Second, the influence of disease severity, phenotype and potential confounders was investigated thoroughly. Third, exhaled breath was obtained within 24 hours after ICU-admission, in the early phase of disease. Finally, we reported these findings strictly following STARD-guidelines to allow for optimal assessment of bias (Table E1).

Nevertheless, several limitations should also be noted. First, the consensus criteria for ARDS are excellent for epidemiological research but may not entirely cover the underlying pathophysiology as meant to be identified using biological markers. Tissue histopathology as a gold standard would have been preferable, but understandably we could not obtain that in this study. Correlation with other markers of ARDS could have added to our understanding, but the design of this study did not allow additional sampling of blood or to perform broncho-alveolar lavages. Future studies could correlate breath profiles with changes in markers of ARDS. Second, we included only mechanically ventilated patients. Therefore, the generalizability of our results to spontaneous breathing patients remains
to be established. Additional studies are also needed to evaluate the clinical application of exhaled breath analysis for biological monitoring of ARDS progression or resolution and to evaluate the influence of previous intubations for mechanical ventilation, like development of lung injury [50] or airway colonization [51, 52]. Third, we found that cardiopulmonary edema and unilateral pneumonia patients could be distinguished from ARDS patients. However, the number of included patients with these diagnoses was very low. This may be explained by the rapid increase of non–invasive mechanical ventilation as an alternative for intubation in these patients. Therefore, although we know that the exhaled breath signal is different in patients between pneumonia or CPE and ARDS, the accuracy of discriminating these patients cannot be quantified precisely.

The prevalence of ARDS was around 40% in our study, which is surprisingly high compared to previous reports [53]. Several factors contribute to this finding. First, ventilated post-surgical patients are not admitted to the ICU routinely in our hospital and when they are admitted because of complications, ARDS is more likely to develop. Second, the included population was severely ill, as exemplified by the high APACHE II score and high mortality. Third, we assessed ARDS prospectively, which could have resulted in the diagnosis of ARDS in patients that would have been missed retrospectively.

The present data suggest that exhaled breath analysis may be used to diagnose ARDS. This study represents a required step towards use of exhaled breath analysis into clinical practice [24]. Next, the predictive value of the observed biological markers should be evaluated in a prospective study including patients that are at risk for ARDS, possibly also including spontaneous breathing patients. Subsequently, the diagnostic accuracy of the combination of risk factors and multiple biological markers should be further improved. In the long run, a move from the diagnostic accuracy paradigm towards a test validation paradigm might be justified [54]. This would allow for the comparison of added value of several index–tests, including exhaled breath analysis, in clinical decision–making. Finally, a bedside test should be developed especially for the detection of VOCs, plausibly octane, acetaldehyde and 3–methylheptane, in exhaled breath of ICU–patients. An array of sensors or rapid mass–spectrometric techniques may be used for that [13]. This may also allow for continuous

**Conclusion**

Exhaled breath analysis showed good diagnostic accuracy for diagnosing ARDS. Discrimination with controls was reproducible and externally validated. The exhaled breath diagnosis of ARDS was not influenced by severity of illness, ventilator settings or comorbidities. Notably, the diagnostic accuracy increased when the exhaled breath analysis was combined with the LIPS. These data suggest that exhaled breath analysis qualifies for the diagnostic assessment of ARDS.

**Online supplement**

For the *online supplement*, please use the following link, or scan the 2D code with your mobile phone.

http://erj.ersjournals.com/content/suppl/2014/04/17/09031936.00005614.DC1/Bos_ERJ_GCMSforARDS_SupplementaryMaterial_R1_1.pdf

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Part III

Diagnosis of Pneumonia
Chapter 7.

Volatile metabolites of pathogens – a Systematic review

Plos Pathogens 2013

Lieuwe DJ Bos, Peter J Sterk & Marcus J Schultz
Abstract

Ideally, invading bacteria are detected as early as possible in critically ill patients: the strain of morbific pathogens is identified rapidly, and antimicrobial sensitivity is known well before start of new antimicrobial therapy. Bacteria have a distinct metabolism, part of which results in the production of bacteria-specific volatile organic compounds (VOCs), which might be used for diagnostic purposes. Volatile metabolites can be investigated directly in exhaled air allowing for non-invasive monitoring. The aim of this review is to provide an overview of VOCs produced by the six most abundant and pathogenic bacteria in sepsis, including Staphylococcus aureus, Streptococcus pneumoniae, Enterococcus faecalis, Pseudomonas aeruginosa, Klebsiella pneumoniae and Escherichia coli. Such VOCs could be used as biological markers in the diagnostic approach of critically ill patients.

A systematic review of existing literature revealed 31 articles. All 6 bacteria of interest produce isopentanol, formaldehyde, methyl-mercaptan and trimethyl-amine. Since humans do not produce these VOCs they could serve as biological markers for presence of these pathogens. The following volatile biomarkers were found for identification of specific strains: isovaleric acid and 2-methyl-butanal for Staphylococcus aureus, 1-undecene, 2,4-dimethyl-1-heptane, 2-butanone, 4-methyl-quinazoline, hydrogen-cyanide and methyl-thiocyanide for Pseudomonas aeruginosa and methanol, pentanol, ethyl-acetate and indole for Escherichia coli. Notably, several factors that may effect VOC production were not controlled for, including used culture media, bacterial growth phase and genomic variation within bacterial strains.

In conclusion, VOCs produced by bacteria may serve as biological markers for their presence. Goal-targeted studies should be performed to identify potential sets of volatile biological markers and evaluate the diagnostic accuracy of these markers in critically ill patients.
Introduction

Sepsis is increasingly prevalent in the developed world comprising 240 per 100,000 persons per year [1]. Early start of targeted antibiotics lowers mortality [2]. However, in the majority of cases empirical antibiotic treatment is untargeted due to inadequate diagnostics, resulting in a three-fold increase in mortality when compared to targeted antibiotic treatment [3].

Ideally, invasion of morbific pathogens is detected as early as possible; the strain of the causative pathogens is identified swiftly, and antimicrobial sensitivity is rapidly known, preferably before start of antimicrobial therapy. However, cultures may take days to become positive and have limited sensitivity, especially in patients already receiving antibiotics because of a previous infection [4]. In addition, contamination could lead to false-positive results and therefore may increase prescription of unnecessary antibiotics [5]. Gram-stain results and direct cellular examination (e.g. of broncho-alveolar lavage fluid) are rapidly available but have limited sensitivity and specificity, and do neither tell the exact strain of pathogen nor its antimicrobial sensitivity [6, 7]. These disadvantages also apply to several biomarkers (c-reactive protein, pro-calcitonine, pro-adrenomedullin and endotoxin) [8-10]. PCR-based diagnostics are currently under investigation and although the results are promising, PCR takes hours before results are available and is laborious and costly [11].

In ancient times, physicians relied heavily on their senses before sophisticated analytical techniques became available. Color, taste, and smell were used to detect biological markers [12]. Bacteria are known to have a characteristic smells. Bacterial strains have a distinct metabolism, part of which results in the production of bacteria-specific volatile organic compounds (VOCs) [13-15]. The metabolic pathways have been described for bacteria in several excellent review articles [14, 15]. However, reviews not yet focused on pathogens and clinical problems.

Detection and identification of VOCs using sophisticated technology may have diagnostic value in medicine [14, 16, 17]. These techniques include gas-chromatography and mass spectrometry (GC-MS), selected-ion flow tube mass-spectrometry (SIFT-MS) [18], ion-molecule reaction mass-spectrometry (IMR-MS) [19, 20] and electronic noses (eNoses)
[16, 21]. GC–MS is used as a gold–standard for separation, detection and identification of VOCs. SIFT–MS and IMR–MS allow for real–time measurement of some VOCs. eNoses do not identify VOCs but rely on pattern–recognition [16]. Volatile compounds can be investigated in \textit{in–vitro} (in culture media or directly in patient material) or directly in the exhaled air (\textit{in–vivo}) allowing for non–invasive monitoring.

Three goals could be pursued with VOC–detection: (1) proof absence of bacterial pathogens (i.e., very high sensitivity and negative predictive value, and therefore no start of antibiotic treatment), (2) identify the presence of a specific strain of bacteria (i.e., very high specificity and positive predictive value, and thus start of appropriate antimicrobial therapy), and (3) separation between phenotypes within bacterial species and therefore prevention of start of antibiotics for which the causative pathogens are not sensitive. However, before VOCs can be tested for these goals in clinical trials, possible diagnostic targets per goal should be known. Therefore, the aim of this review is to provide an overview of volatile organic compounds produced by the six most abundant and pathogenic bacteria in sepsis: \textit{Staphylococcus aureus} (SA), \textit{Streptococcus pneumoniae} (SP), \textit{Enterococcus faecalis} (EF), \textit{Pseudomonas aeruginosa} (PA), \textit{Klebsiella pneumoniae} (KP) and \textit{Escherichia coli} (EC) [22].
Methods

A broad systematic search in the EMBASE library was performed on the 1st of August 2012 using the following terms: “(mass and spectrometry) and bacteria and volatile”.

Articles were selected for full-text examination if the title and/or abstract suggested the investigation of bacterial pathogens in a clinically relevant setting and the measurement of volatile organic compounds.

Selected articles were read and included if (a) one or more of the following, most frequently cultured pathogens on the ICU [22], was investigated: Staphylococcus aureus, Streptococcus pneumoniae, Enterococcus faecalis, Pseudomonas aeruginosa, Klebsiella pneumoniae or Escherichia coli and (b) a summary of detected volatile organic compounds per pathogen was provided. Furthermore, all references of the selected articles were scanned based on title and selected based on the previous criteria. Double publications of the same data were disregarded.

All volatile organic compounds described in the included articles were summarized in 9 tables (see supplemental information) based on the following molecular structures (adapted from Hakim et al. [64]): hydrocarbons, alcohols, acids, aldehydes, ketones, cyclic compounds, esters, S–containing and N–containing. They were referred to in the text by number (#). If a molecule could be included in more than one table, the most appropriate category was chosen to avoid duplicates.

The production of a VOC by a pathogen in an article was indicated with a “+” and the absence of a molecule with a “−”. The results section focuses on metabolites found in more than one study. The rows of these metabolites also received a coloring in tables S1–S9 based on level of evidence for absence, or presence of a metabolite. Cells were colored based on the pooled results for a VOC per pathogen, for all included studies. A clear cell indicated there is little evidence (zero or one study). When there is convincing evidence a VOC is produced by a pathogen, the cell is colored green (more positive than negative evidence, with more than one study difference). A red cell means that pathogen is not known or rarely found to produce that molecule (more negative than positive evidence, with more than one study difference). Contradicting evidence resulted in an
orange cell.

**Results**

The MEDLINE search resulted in 837 articles of which 778 were excluded based on title and/or abstract (figure 1). Fifty–nine articles were read and tested for inclusion criteria. This resulted in the inclusion of twenty–seven articles (table 1). Ten articles were read based on references, of which 4 were included, bringing the sum of included articles to thirty–one.

The articles originated from 1977 to 2012, with a rapid increase in number of publications from 2006. Fifteen articles reported on data collected with GC–MS, 7 on data collected with SIFT–MS, three with IMR–MS, and six with other techniques. Seven studies used clinical samples; twenty studies used reference strains. Results on 161 metabolites were obtained of which a minority was studied in multiple papers. The findings are reported in tables S1 to S9 and summarized below, per functional group. The most prominent VOCs and their (cross-)association with the six selected gram positive and gram negative bacteria are illustrated in figure 2.

**Figure 1: Flowchart**

![Flowchart diagram]
Table 1: Literature overview

<table>
<thead>
<tr>
<th>Year</th>
<th>1st Author</th>
<th>Pathogen</th>
<th>Method</th>
<th>Remarks</th>
<th>Ref.</th>
</tr>
</thead>
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<td>Hayward</td>
<td>SA, PA, EC</td>
<td>GLC</td>
<td>Through references</td>
<td>[65]</td>
</tr>
<tr>
<td>1979</td>
<td>Cox</td>
<td>PA</td>
<td>GC + Colometric</td>
<td>Through references</td>
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</tr>
<tr>
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<td>Labows</td>
<td>PA</td>
<td>GC−MS</td>
<td>Pathway description</td>
<td>[39]</td>
</tr>
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<td>SA, PA, EC</td>
<td>HS−GLC</td>
<td></td>
<td>[67]</td>
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<td>Zechman</td>
<td>SA, PA, KP</td>
<td>GC−MS</td>
<td>Pathway description</td>
<td>[28]</td>
</tr>
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<td>Kuzma</td>
<td>PA, EC</td>
<td>GC−MS</td>
<td></td>
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<tr>
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<td>PA</td>
<td>GC−FID</td>
<td></td>
<td>[68]</td>
</tr>
<tr>
<td>2000</td>
<td>Julák</td>
<td>SA, SP, EF, PA, KP, EC</td>
<td>GC−MS</td>
<td>Clinical samples</td>
<td>[69]</td>
</tr>
<tr>
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<td>Julák</td>
<td>SA, SP, EF, PA, KP, EC</td>
<td>GC−FID</td>
<td>Clinical samples</td>
<td>[53]</td>
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<tr>
<td>2005</td>
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<td>PA</td>
<td>SIFT−MS</td>
<td>Through references</td>
<td>[70]</td>
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<td>2005</td>
<td>Hamilton–Kemp</td>
<td>EC</td>
<td>GC−MS</td>
<td>Through references</td>
<td>[71]</td>
</tr>
<tr>
<td>2006a</td>
<td>Allardyce</td>
<td>SA, SP, PA, EC</td>
<td>SIFT−MS</td>
<td>Antibiotic effects</td>
<td>[56]</td>
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<tr>
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<td>Allardyce</td>
<td>SA, SP, PA, EC</td>
<td>SIFT−MS</td>
<td>Two different timepoints</td>
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<td>Julak</td>
<td>PA</td>
<td>SIFT−MS</td>
<td>Clinical samples</td>
<td>[52]</td>
</tr>
<tr>
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<td>Scotter</td>
<td>SA, SP, PA, EC</td>
<td>SIFT−MS</td>
<td></td>
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<td>Bunge</td>
<td>EC</td>
<td>PTR−MS</td>
<td>Different timepoints</td>
<td>[73]</td>
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<td>Syhre</td>
<td>SA, SP, EC</td>
<td>GC−MS</td>
<td>Clinical samples</td>
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<td>MCC−IMS + GC−MS</td>
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<td>Preti</td>
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<td>GC−MS</td>
<td>Clinical samples</td>
<td>[40]</td>
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<td>SIFT−MS</td>
<td>Multi−variate analysis</td>
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<tr>
<td>2010</td>
<td>Zhu</td>
<td>SA, PA EC</td>
<td>SESI−MS</td>
<td></td>
<td>[75]</td>
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<td>2010</td>
<td>Chambers</td>
<td>SP</td>
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<td>Savelev</td>
<td>PA</td>
<td>GC−MS</td>
<td>Clinical samples</td>
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<tr>
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<td>Shestivska</td>
<td>PA</td>
<td>GC−MS</td>
<td></td>
<td>[76]</td>
</tr>
<tr>
<td>2011</td>
<td>Storer</td>
<td>SA, EF, PA, KP, EC</td>
<td>SIFT−MS</td>
<td>Inoculated urine</td>
<td>[77]</td>
</tr>
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<td>2012</td>
<td>Bean</td>
<td>PA</td>
<td>GC/GC−TOF−MS</td>
<td>Two different timepoints</td>
<td>[78]</td>
</tr>
<tr>
<td>2012a</td>
<td>Dolch</td>
<td>PA, EC</td>
<td>IMR−MS</td>
<td>Two different timepoints</td>
<td>[49]</td>
</tr>
<tr>
<td>2012b</td>
<td>Dolch</td>
<td>SA, EF</td>
<td>IMR−MS</td>
<td>Two different timepoints</td>
<td>[50]</td>
</tr>
<tr>
<td>2012</td>
<td>Filipiak</td>
<td>SA, PA</td>
<td>GC−MS</td>
<td>Different timepoints</td>
<td>[48]</td>
</tr>
</tbody>
</table>

SA = Staphylococcus aureus, SP = Streptococcus pneumoniae, EF = Enterococcus faecalis, PA = Pseudomonas aeruginosa, KP = Klebsiella pneumoniae, EC = Escherichia coli.
Hydrocarbons (table S1)

In table S1, the hydrocarbons investigated in pre-specified pathogens are listed. One of the most investigated hydrocarbons is isoprene [#13], which seems to be produced in both Gram-positive and Gram-negative bacteria, although studies show contradicting results on their presence. The production is most likely through the methylerythritol phosphate pathway and is growth dependent (high during the log-phase and low during the stationary phase) and occurs primarily in a nutrient rich environment [23, 24]. Isoprene is also one of the main volatiles in the breath of mammals and is less applicable as focus for in-vivo studies [25]. 1-Undecene [#6] and other less well-studied alkenes [#3-5] are suggested to be produced mainly by PA, and are most likely the product of degradation of fatty acids through the B-oxidation pathway, a pathway that is suggested for most volatile hydrocarbons [15, 26]. 1,3-Butadiene [#2] is reported to be produced by Gram-positive bacteria, but not by Gram-negative bacteria.

Alcohols (table S2)

1-Alcohols are produced through b- or a-oxidation of fatty acid derivates through acetyl-CoA. Ethanol [#30] is one of the most studied volatiles [15]. It can be produced by all investigated bacteria, but some (SA, SP, KP and EC) produce it almost always, while others (EF and PA) have been found to lack ethanol in the headspace. Methanol [#32], propanol [#34], butanol [#27], pentanol [#36] and some longer chain 1-alcohols [#28, 29, 35] are most prominently produced by EC, however not exclusively. EC might use these alcohols to inhibit the growth of other bacteria [27]. The branched alcohol isopentanol [#25] is found less frequently in EC, compared to the other pathogens. This metabolite is produced through another pathway, possibly via isovaleryl-CoA, since concentrations increase when leucine is added to the growth medium [28].

Acids (table S3)

Fatty acids could be a marker for anaerobic metabolism, but are not strain specific [29, 30]. However, anaerobic dependent production does not apply to very short, volatile fatty acids. Acetic acid [#37] is most frequently produced by SA, but also by the other pathogens. Isovaleric acid [#42]
is more exclusively produced by SA. Propionic acid [44] has only been reported in the headspace of KP. Other acids [38–41, 45] have not been identified in the headspace of the pathogens studied in that review.

**Aldehydes (table S4)**

Formaldehyde [56] is produced by many bacteria [31], including the six species on which we focus in this review. Acetaldehyde [54] is also produced by most pathogens, but PA and KP are less likely to produce this metabolite. Notably, acetaldehyde and benzaldehyde [55] are known to have antimicrobial activity [32]. Methylpropanal [49], 3–methyl–butanal [53] and 2–methyl–butanal [48] are modifications of amino–acids and intermediates for the formation of many ester and branched ketones [15]. Methyl–propanal and 2–methyl–butanal are mostly produced by SA while methylpropanal is produced by all investigated pathogens.

**Ketones (table S5)**

Methyl–ketones are produced during decarboxylation of fatty acid derivates. The smallest, acetone [81], is produced by most bacteria, but not under all circumstances. Furthermore, acetone is also present in high concentrations in breath, limiting the in–vivo applicability as a biomarker for bacterial presence. The longer 2–ketones [62–70] are classically biomarkers for PA [28], but the pooled results provide evidence only for 2–nonanone, 2–dodecanone, 2–pentanone and 2–heptanone. 2–Nonanone is also produced by SA. Acetoin or 3–hydroxybutanone [79] is used to differentiate between lactose fermenting and nonfermenting Enterobacteriaceae. Surprisingly, in one study acetoin was found in the headspace of the non–fermenting EC. This suggests the involvement of other pathways for the production of acetoin [33]. In SA, acetoin generation has been linked to murein hydrolase activity, stationary–phase survival and antibiotic resistance [33].

**Cyclic compounds (table S6)**

2–Phenylethanol [87] is one of the most widespread microbial VOCs [15], but not in the hereby investigated pathogens. 2–Pentylfuran [86] has been proposed as a biomarker for Aspergillus [34, 35] but was also found in the headspace of SP. Two pathways of production via linoleic
acid have been proposed: enzymatically controlled oxidation and direct interaction with reactive oxygen species [34]. Limonene [#89], phenol [#92] and toluene [#93] are identified as potential markers for bacterial presence in this study. This might imply that earlier statements that these compounds should be regarded as exogenous when found in the patients’ breath need reconsideration [36]. In a study by Holland, germfree rats were compared to conventional rats [37]. Urinary acetophenone [#88] was 13-fold increased in conventional rats, suggesting bacterial production of this compound. This was indeed reported in one in-vitro study. Several other compounds were found in conventional rats of which 4-heptanone [#76], 2-heptanone [#66] and 5-methyl-2-hexenal were the most significant and are also produced by bacteria in-vitro.

**Esters (table S7)**

Ethyl acetate [#100] and other acetate containing esters [#99, 108] are the product of esterification between acetic acid and a fatty acid. However, the pathogens producing most acetic acid, such as SA are not the same as the ones with the most prominent ethyl acetate production, such as EC. The factors influencing this reaction remain unknown. Ethyl butanoate can be produced by all six pathogens, but is mostly found in *Enterococcus* and EC.

**S-containing (table S8)**

The most important volatile sulfur containing organic compounds are hydrogen-sulfide [#120], methyl-mercaptan [#122], dimethylsulfide [#118], dimethyldisulfide [#117] and dimethyltrisulfide [#119]. All are highly toxic and might be involved in the induction of inflammation [38]. All bacteria are able to produce these compounds but they might provide additional information about the species of pathogen. Hydrogensulfide is produced mainly by EF and EC, while dimethyldisulfide is more frequently found in the gram negative bacteria. Dimethyltrisulfide might be a marker for PA and dimethylsulfide for PA and SP.
The six investigated pathogenic bacteria are plotted. All the metabolites for which convincing evidence on production by at least one of the bacteria was available were included in the figure and connected with a line to all bacteria known to produce a particular metabolite. Four zones of interest are highlighted. The blue zone in the middle indicates metabolites that are (almost) always produced by all pathogens and are therefore candidate markers with a high sensitivity that might thus qualify for the exclusion of infection (high negative predictive value). The three red zones indicate metabolites that are produced by only or mainly one strain of bacteria; these are possibly volatile biomarkers specific for a pathogen with a very high positive predictive value.
N−containing (table S9)

The simplest N−containing volatile organic compound, ammonia [142], is most frequently produced by SA and PA. Hydrogencyanide [147] is only investigated in cultures of Pseudomonas, but was found to be produced in all studies. Trimethyl−amine [161] might be a marker for PA and EC. 2−Aminoacetophenone (2−AA) [130] was recently proposed as a breath marker for PA and is responsible for the grape−like odour associated with PA infections [39, 40]. However, review of the literature clearly shows it can be produced by most bacteria and is frequently found in SP and EC. Furthermore, 2−AA can be found in a variety of food products and after consumptions found in exhaled breath, resulting in false−positive results [41]. Indole [148] is a direct product of deaminating L−tryptophan by tryptophanase. It is mainly produced by EC, but it is sporadically detectable in the headspace of other pathogens. Tryptophanase is essential for biofilm formation, thus indole can be regarded as a biomarker for this bacterial phenotype [42, 43].

Discussion

Pathogenic bacteria are capable of producing a large variety of volatile metabolites. Our systematic review identified thirty−one articles reporting on VOC production by the most important pathogens of sepsis. However, only a very small fraction of the metabolites is produced exclusively by one of the bacterial species of interest. Notably, some studies failed to replicate the results of previous experiments resulting in contradicting overall results. Despite these limitations, several sensitive and some very specific candidate biomarkers were identified by systematically summarizing the available literature (figure 2).

The large number of contradicting results between the studies might be explained by four variables. Firstly, not all studies used the exact same subtype of bacterial species. In one study, phage types of SA influenced headspace volatile organic compounds [44]. Genomic variation between subtypes could result in differences in efficacy of enzymes within a specific metabolic pathway. These variations might be useful to phenotype within species of bacteria, though. However, this could hamper the clinical applicability of volatile biomarkers for strain identification. Secondly, the
growth medium is the source of building blocks for the produced VOCs and therefore a confounding variable [44-47]. Thirdly, measurements were obtained at different time-points in the growth of bacteria. Several studies investigated this phenomenon and found that depletion of metabolites and growth phase (log or stationary) influence headspace metabolites [48-51]. Lastly, the majority of the included studies investigated cultures of reference strains. However some studies focused on clinical samples, in which within-class variation was increased [52-54]. Patient samples are less well defined than laboratory produced cultures of reference strains and are different in the following aspects: CFU’s, growth phase, host response, viscosity [55], confounding co-morbidities and medications (e.g. antibiotics [56]).

Several biomarkers qualify for clinical investigation with regard to the first goal of biomarker research; proof absence of a bacterial pathogen. Isopentanol, formaldehyde, methyl–mercaptan and trimethyl–amine are produced by all bacteria and not by the host (blue area in figure 2). Ethanol and Isoprene are also sensitive candidate markers but are found in large quantities in breath of mammals. If the aim of a study is to exclude bacterial infection from the differential diagnosis, a set of volatile biomarkers with a high a-priori chance of being produced by a lot of pathogens should be investigated. Not finding any of these candidate markers might have a high negative predictive value.

Identification of specific strains might be performed using the following VOCs: SA – isovaleric acid and 2–methyl–butanal; PA – 1–undecene, 2,4–dimethyl–1–heptane, 2–butanone, 4–methyl–quinazoline, hydrogen–cyanide and methyl–thiocyanide; EC – methanol, pentanol, ethyl–acetate and indole (red areas in figure 2). No candidate biomarkers for SP, EF and KP could be identified in the literature yet. For the identification of species of pathogens, a combination of volatile organic compounds is recommended. The advantages of this approach are illustrated in a recent paper by Thorn and in several studies using electronic nose technology [13, 55, 57, 58]. It is imperative that diagnostic accuracy (sensitivity and specificity) is reported following the STARD guidelines [59], as is only done in one of the studies included in this review [60].
Phenotyping of pathogens should focus on infecting/colonizing bacteria, bacterial growth and bacterial resistance. Interestingly, indole was found to be a biomarker for biofilm formation in EC and might thus be used to separate between clinically relevant phenotypes within the same strain of bacteria. Secondly, small volatile sulfide–containing organic compounds were found to induce inflammation in a rat model and might thus serve as a marker for pathogenicity. Thirdly, the production of several VOCs is decreased after the addition of antibiotics in levels above MIC to the culture medium, suggesting that therapeutic response can be monitored. Antibiotic administration below MIC did lower VOC concentrations, but to a lower extend, suggesting a dose dependency. The influence of bacterial resistance on VOCs was not described in the included papers. However, the first steps in this direction are taken in a recent paper on colometric electronic nose technology discriminating methicillin–resistant SA from methicillin–sensitive SA and vancomycin–resistant EF from vancomycin–sensitive EF [58].

This review has several limitations. First of all, most included studies did not include all pre–selected pathogens and thus provide partial evidence for clinical questions involving all pathogens. Secondly, since most studies did not report quantitative measures and used different sampling techniques no headspace concentrations could be given per compound per study. Thirdly, increased headspace concentrations were reported but decreased concentrations may have been missed in some studies. Indeed, the absence of a normally present metabolite might be just as much proof of the presence of a pathogen as the presence of another VOC. Finally, different technologies were used to detect the volatile organic compounds. GC–MS was mostly used, although not always with the same materials and separation methods. However, while keeping the limitations of the used separation methods in mind, GC–MS remains the gold standard for volatile organic compound discovery. In this review, we also included studies using SIFT–MS, IMR–MS and other techniques that allow for compound identification. These technologies are not as powerful as GC–MS in separating and identifying metabolites but were nevertheless included in this review since they did allow for identification of some compounds.
If volatile organic compounds are used *in-vivo* for diagnostic purposes in sepsis, several considerations must be taken into account. First of all, the growth medium inside the host might be entirely different from *in-vitro* growth media, resulting in a different set of produced metabolites. Secondly, the host will interact with the bacteria through an inflammatory response, which might alter metabolism. This inflammatory response can alter human metabolism in itself and future studies will need to address the metabolomic difference between and an infectious and a non-infectious inflammatory response [25, 61]. Thirdly, VOCs can be derived from diet and environment. Finally, the body, including the lung, is host to a unique microbiome, even in healthy conditions [62, 63]. It might very well be that these residential bacteria produce similar metabolites and therefore interfere with a VOC-based diagnostic test. In this scenario, VOCs altered by inflammation might be used to further discriminate between colonizing and pathogenic bacteria.

In conclusion, several volatile biomarkers show to be particularly promising candidates for proof of absence of infection, whereas some others qualify for the detection of bacteria and identification of the six investigated bacterial species. However, only a limited amount of research is available. Therefore, targeted studies should be performed to identify potential sets of volatile biomarkers and evaluate the diagnostic accuracy of these markers in critically ill patients.

**Online Supplement**

For the online supplement, containing supporting information please follow this link: files.figshare.com/1058279/Text_S1.doc or access through the 2D-code below.
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Chapter 8.

Volatile Metabolic Fingerprint of Ventilator-associated Pneumonia

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Abstract

There is urgent need for a better diagnostic approach of ventilator–associated pneumonia (VAP). Invading respiratory pathogens as well as patients produce volatile organic compounds (VOCs) that could serve as early biological markers for VAP. We hypothesize that volatile metabolic fingerprints can discriminate airway samples of patients with VAP from those of patients without VAP.

In this prospective cohort study, VOCs in headspace of tracheal aspirates from intubated and ventilated intensive care unit patients were analysed using electronic nose technology (eNose).

The analysis included 45 patients, of which 14 were classified as having ‘VAP’, 14 as ‘colonized patients without VAP’ and 17 as ‘non–colonized patients without VAP’. eNose analysis discriminated between VAP and patients without VAP with an area under the receiver operating characteristic curve (ROC−AUC) of 0.85 [95%–confidence interval (CI): 0.69–1.0]. Discrimination was not influenced by colonization of the airways in patients without VAP (p = 0.42). VOC fingerprinting of airway samples improved diagnostic accuracy for VAP on top of the clinical pulmonary infection score (net classification improvement: 1.4 [CI: 0.80–2.07, p<0.001]). Longitudinal analysis showed that the slope of the eNose signal was steeper in patients that went on to develop VAP (0.04 vs. 0.002, p=0.009).

VOCs are promising biological markers for the diagnosis of VAP. Diagnostic accuracy does not seem to be influenced by colonization of airways. Longitudinal analysis showed that the slope of the eNose signal is also different in patients that develop VAP.
**Introduction**

Ventilator–associated pneumonia (VAP) is a frequent complication in intubated and mechanical ventilated critically ill patients [1, 2]. The diagnosis remains complex as radiological confirmation of a new or progressive pulmonary infiltrate and symptoms of pulmonary inflammatory response are sensitive for VAP but a more definitive diagnosis requires positive microbiological cultures [3-5]. However, it may take days before the culture results become available and they could be false–negative [6]. Thus there is urgent need for a novel approach of VAP early diagnosis. Importantly, the diagnostic process should not only detect respiratory pathogens in the airways, but also differentiate infection from airway colonization, which is frequent in intubated patients [7].

In ancient times, physicians relied heavily on their senses for disease detection. Colour, taste, and smell were used to detect biological markers [8]. Bacterial strains are known to have a characteristic smell produced by volatile metabolic end–products of bacteria–specific biochemical pathways called volatile organic compounds (VOCs) [9-13]. The host interacts with bacteria through an oxidative and inflammatory response, which could even modulate other bacterial metabolic pathways. Certainly, this host response alters human metabolism in itself [14, 15]. VOCs, whether produced by invading pathogens or by the host, can be detected in the headspace of liquid airway samples (e.g., lavage fluids) or exhaled breath [16]. Such VOC–profiles have been shown to correlate with the frequently used clinical pulmonary infection score (CPIS) [17] and could be useful in discriminating between different pathogen species [18-20] but have not been compared to the consensus diagnostic criteria for VAP [21].

We hypothesize that VOC–profiles from airways samples can identify airway samples of intubated and ventilated patients with VAP. To test this hypothesis we included patients that did and did not develop VAP. VOC–profiles were detected in tracheal aspirates (TA) using a sensor based technology called electronic nose (eNose) [22]. This technology can be used at the bedside and provides a result within minutes [22]. We analysed whether identification of VAP by VOC–profiles depends on colonization of the airways, investigated the influence of the amount and Gram-status of bacteria in the tracheal aspirate, quantified the complementary value
of VOCs to CPIS and explored the development of the eNose signal over time.

**Methods**

*Design and setting*

This is a study within an international multi-center prospective observational cohort that evaluated the predictive value of biological markers for development of VAP. The study protocol was reviewed and approved by the Medical Ethical Committee of Parc Tauli, Sabadell, Spain (IRB: 2008/524).

*Inclusion and exclusion criteria*

Inclusion criteria were: (1) recruited to one of the participating ICUs, (2) intubated and ventilated for another reason than pneumonia, and (3) expectation that mechanical ventilation was needed for longer than 48 hours. Exclusion criteria were: (1) age less than 18 years, (2) expectation that withdrawal of treatment could happen within 72 hours, and (3) pregnancy or lactation. Furthermore we excluded patients who received antimicrobial therapy within the last 5 days before ICU admission (prophylactic antimicrobial therapy, e.g., for surgical procedures, was allowed), and patients with fulminant hepatic failure, pancreatitis, or disseminated cancer, since these all could effect levels of biomarker of interest in the original study. Finally, for the purpose of the present analysis we also excluded patients who developed pneumonia within the first two days in the ICU.

*Diagnostic definitions and patient selection*

Patients were classified into three groups (table 1). VAP was diagnosed using consensus criteria [21] (a new and persistent radiographic infiltrate plus at least 2 of the following criteria: a) temperature > 38°C or < 36°C; b) leucocytes >10 or <4 x 10³/mm³; c) purulent tracheal aspirate [23, 24]) but always needed microbiological confirmation to fulfill the diagnosis of ‘VAP’ (> 10³ or ≥ 10⁶ colony forming units (CFU)/ml in mini-bronchoalveolar lavage (BAL) fluid or tracheal aspirate (TA), respectively). Patients not fulfilling the abovementioned criteria for VAP but of whom
microbiological culture revealed presence of bacteria in mini-BAL or TA were classified as ‘colonized patients without VAP’. Patients not fulfilling the abovementioned criteria for VAP with negative cultures were classified as ‘non–colonized patients without VAP’.

Data and sample collection

Patient demographics, primary (and admission) diagnosis, SAPS II [25], APACHE II score [26] and ICU mortality were recorded for all patients. Tracheal aspirates (TA) were collected on Mondays, Wednesdays and Fridays and were sent for quantitative culture. Mini-BAL was performed on the day of clinical suspicion of VAP. Isolates were characterized by colony morphology and Gram stains. The remaining portions of TA samples were saved at −80°C (figure 1, bullet 1).

Figure 1: Plan for sample selection and statistical analysis
Headspace analysis

TA fluid samples were defrosted and subsequently analysed using an eNose (figure 1, bullet 2). For this, 0.5 ml supernatant (1500 rpm for 15 minutes at 4°C) of TA fluid was transferred into a 5 ml headspace vial (MN–net N 20–5 DIN, clear with crimp top, Fisher Scientific, Landsmeer, the Netherlands), topped with a 20 mm headspace cap (Fisher Scientific, Landsmeer, the Netherlands) and warmed to room temperature. Two needles were inserted into the headspace cap before start of each measurement. One needle was placed into the sample and was purged with pure nitrogen gas (99.9999%, Linde Gas, Dieren, the Netherlands). The second needle was placed in the headspace for collection of gas (figure S1).

We used the Cyranose 320 eNose (Smith Detections, Pasadena, CA) containing a nano–composite sensor array with 32 polymer sensors (figure 1, bullet 3) [22]. This electronic nose relies on semi-selective recognition of VOCs as each sensor is cross-reactive to a variety of functional chemical groups. The combined response of the sensor array can be used for pattern-recognition and disease classification [27]. A full description of the chemical and statistical rationale for this approach is outside the scope of the present paper and has recently been discussed in an excellent review by Konvalina and Haick in Accounts of chemical research and is summarized in the online supplement [28]. Nitrogen gas was sampled for 30 seconds as a baseline-measurement, where after gas from the headspace was analysed for one minute using a low flow (40 ml/min). Afterwards, the eNose was purged to let the sensors recover to baseline. This was done in duplicate.

Statistical analysis

Differences between the groups were compared using the Mann–Whitney U test for continuous variables and chi–square for categorical variables. Data were summarized using the median and inter-quartile range for continuous variables and with count and percentage for categorical variables. All analyses were performed in R statistics using R studio [29]. P–values below 0.05 were considered significant.
Samples from patients with VAP were to be compared in time to those from patients who did not develop VAP. For this, we first determined the day the diagnostic criteria for VAP were met in patients who developed VAP. This was day 7 after ICU admission. We then selected patients who did not develop VAP, but who were still intubated and ventilated on day 7 after ICU admission to create the control group. To train the diagnostic algorithm ‘VAP’ patients were considered cases and both ‘colonized patients without VAP’ and ‘non–colonized patients without VAP’ were taken together as controls.

Sparse partial least square (SPLS) logistic regression was used to produce a diagnostic model (figure 1, bullet 4). SPLS analysis is a suitable form of regression that can select predictive variables and limit false discovery in situations were large number of independent variables are investigated in low numbers of individuals [30]. Two parameters are set in SPLS: K and eta. These were tuned using 10-times cross-validation [30]. SPLS logistic regression resulted in a predicted probability of VAP, which is called the “eNose signal” in this manuscript and was used for further analyses. Receiver operator characteristics (ROC) analysis was performed and the area under the curve (AUC) was reported (figure 1, bullet 5).

A sensitivity analysis was performed for ‘colonized patients without VAP’ and ‘non–colonized patients without VAP’ (figure 1, bullet 6). Secondly, we investigated the correlation between the eNose signal and to the number of colonizing forming units using Spearman’s correlation. Thirdly, the diagnostic performance of the eNose logistic regression function was compared to the CPIS and the net classification improvement of the combination of the two diagnostic tests was assessed [31]. Finally, the development of the eNose signal was displayed over time using a LOESS smoother and analyzed by means of mixed-model analysis. The period preceding VAP (7 days before diagnosis) was investigated (figure 1, bullet 7). The slope of the eNose signal was fitted using a linear mixed model with a random intercept and a random slope for the eNose signal per patient, as described previously [32]. The slopes were compared between patients with and without VAP and the area under the ROC-curve was calculated.
Results

Patients

Figure 2 shows the CONSORT diagram of the study; of 154 eligible patients, 10 development of pneumonia within 48 hours, and from 27 patients too much data was missing. Of the remaining 117 patients 14 patients fulfilled the diagnostic criteria for VAP (on median day 7); 103 patients never met the diagnostic criteria for VAP, and of them only 31 patients were intubated and ventilated for longer than 7 days. These patients served as controls in the planned analysis. Of the patients without VAP, 14 had colonized airways. Table 1 shows the patient characteristics and microbiological data. There were no missing physiological data (ventilator setting, hemodynamics, temperature) and follow–up for mortality was available for all patients in the database. None of the patients were treated with inhaled antibiotics or systemic corticosteroids.

Figure 2: Patient inclusion
Table 1: Patient characteristics

<table>
<thead>
<tr>
<th></th>
<th>No VAP (n=31)</th>
<th></th>
<th>VAP (n=14)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>non–colonized</td>
<td>colonized</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(n=17)</td>
<td>(n=14)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, yrs N (%)</td>
<td>61 (33)</td>
<td>59 (31)</td>
<td>45 (24)</td>
<td>0.20</td>
</tr>
<tr>
<td>Male N (%)</td>
<td>15 (48)</td>
<td>8 (57)</td>
<td>7 (50)</td>
<td>0.89</td>
</tr>
<tr>
<td>APACHE II median (IQR)</td>
<td>20 (10)</td>
<td>20 (13)</td>
<td>24 (7)</td>
<td>0.11</td>
</tr>
<tr>
<td>SAPS II median (IQR)</td>
<td>55 (20)</td>
<td>60 (19)</td>
<td>52 (27)</td>
<td>0.48</td>
</tr>
<tr>
<td>COPD N (%)</td>
<td>2 (6)</td>
<td>2 (12)</td>
<td>0 (0)</td>
<td>0.91</td>
</tr>
<tr>
<td>Respiratory failure N (%)</td>
<td>4 (13)</td>
<td>1 (8)</td>
<td>3 (21)</td>
<td>0.58</td>
</tr>
<tr>
<td>Shock N (%)</td>
<td>4 (13)</td>
<td>3 (21)</td>
<td>1 (8)</td>
<td>4 (29)</td>
</tr>
<tr>
<td>Low consciousness N (%)</td>
<td>17 (55)</td>
<td>8 (57)</td>
<td>9 (75)</td>
<td>6 (43)</td>
</tr>
<tr>
<td>Other N (%)</td>
<td>6 (19)</td>
<td>3 (21)</td>
<td>3 (21)</td>
<td>1 (7)</td>
</tr>
<tr>
<td>No N (%)</td>
<td>17 (55)</td>
<td>17 (100)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>2 (6)</td>
<td>0 (0)</td>
<td>2 (14)</td>
<td>3 (21)</td>
</tr>
<tr>
<td>Pseudomonas aeuginosa.</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Klepsiella pneumoniae</td>
<td>1 (3)</td>
<td>0 (0)</td>
<td>1 (7)</td>
<td>2 (14)</td>
</tr>
<tr>
<td>Haemophilus influenza</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>3 (21)</td>
</tr>
<tr>
<td>Acinetobacter baumannii</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>3 (21)</td>
</tr>
<tr>
<td>Other gram negative</td>
<td>3 (10)</td>
<td>0 (0)</td>
<td>3 (21)</td>
<td>4 (28)</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>6 (19)</td>
<td>0 (0)</td>
<td>6 (43)</td>
<td>2 (14)</td>
</tr>
<tr>
<td>Candida species</td>
<td>4 (13)</td>
<td>0 (0)</td>
<td>4 (29)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Tidal volume (ml)</td>
<td>446 (145)</td>
<td>447 (161)</td>
<td>438 (89)</td>
<td>0.40</td>
</tr>
<tr>
<td>Plateau pressure (cmH2O)</td>
<td>20 (8)</td>
<td>23 (8)</td>
<td>24 (8)</td>
<td>0.40</td>
</tr>
<tr>
<td>Positive end expiratory pressure (cmH2O)</td>
<td>5 (2)</td>
<td>5 (2)</td>
<td>8 (4)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CPIS (day measurement)</td>
<td>3 (4)</td>
<td>3 (4)</td>
<td>11 (2)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>WBC (x103)</td>
<td>10 (4)</td>
<td>10.5 (2.5)</td>
<td>14.1 (8.0)</td>
<td>0.06</td>
</tr>
<tr>
<td>28 day mortality N (%)</td>
<td>4 (13)</td>
<td>1 (8)</td>
<td>6 (43)</td>
<td>0.13</td>
</tr>
</tbody>
</table>

*: P-value for all patients without VAP vs. all patients with VAP.
†: Multiple organisms could have been cultured per patient.
Airway samples

175 airway samples were obtained. The median number of samples per patient was 4 [3 – 6] and 2 [2 – 3] in the VAP group and the groups of patients without VAP, respectively. One sample per patient was used for the cross-sectional statistical analysis (for patients with pneumonia: sample on day of diagnosis, for patients without pneumonia: sample obtained closest to day 7). The median day of sample collection was 7 [5 – 9] and 5 [4 – 8] for patients with VAP and patients without VAP, respectively.

Discrimination between samples of patients with VAP and without VAP

SPLS analysis of eNose data (K = 4, eta = 0.8) resulted in the selection of 26 sensors (for coefficients, see Table S1). Using the eNose model, VAP (median predicted probability: 0.53 [0.44 – 0.90]) and patients without VAP (colonized and non-colonized) (median: 0.14 [0.10 – 0.23]) could be discriminated with a ROC–AUC of 0.85 [CI: 0.69 – 1.0] (table 2).

Sensitivity analysis for colonization in patients without VAP

The eNose predicted probability was not different for patients without VAP, with and without colonized airways: 0.12 [0.09 – 0.22] and 0.14 [0.12 – 0.24], respectively (p = 0.42). VAP was well distinguished from non-colonized and from colonized patients without VAP: ROC–AUC of 0.84 [CI: 0.68 – 1.0] and 0.85 [CI: 0.68 – 1.0], respectively (table 2).

Correlation with bacterial growth

Bacterial growth was associated with a higher eNose signal (Spearman’s correlation coefficient: 0.56, p<0.001; figure S2). When the analysis was limited to samples with bacterial growth, the amount of CFU was not correlated with the eNose signal (Spearman’s correlation coefficient: 0.22, p=0.36, figure S2).

Comparison and combination with CPIS

The ROC–AUC for the CPIS by itself was 0.89 [CI: 0.80 – 0.99] for ‘VAP’ (table 2). The ROC–AUC was 0.95 [CI: 0.88 – 1.0] after combination of
CPIS with the eNose algorithm (table 2). The net classification improvement [31] was 1.4 [CI: 0.80 – 2.07, p < 0.001] for ‘VAP’.

Longitudinal analysis

Figure 3 shows the development of the eNose signal during ICU-stay. The slope of the eNose signal was 0.04 [0.02 – 0.05] for patients with VAP and 0.002 [-0.02 – 0.01] for patients without VAP (p=0.009). Using the slope of the eNose signal VAP and patients without VAP (colonized and non-colonized) could be discriminated with a ROC–AUC of 0.76 [CI: 0.56 – 0.96].

Figure 3: Breathprint over time

![Graph showing the development of the eNose signal for VAP and No VAP patients.](image)
Table 2: Diagnostic accuracy

<table>
<thead>
<tr>
<th>Measure</th>
<th>Control group</th>
<th>ROC-AUC [95% CI]</th>
<th>Cut-off</th>
<th>Sens</th>
<th>Spec</th>
<th>PPV</th>
<th>NPV</th>
</tr>
</thead>
<tbody>
<tr>
<td>eNose</td>
<td>All controls</td>
<td>0.85 [0.69 – 1.0]</td>
<td>0.41</td>
<td>94%</td>
<td>79%</td>
<td>91%</td>
<td>85%</td>
</tr>
<tr>
<td></td>
<td>Non-colonized controls</td>
<td>0.84 [0.68 – 1.0]</td>
<td>0.38</td>
<td>94%</td>
<td>79%</td>
<td>84%</td>
<td>92%</td>
</tr>
<tr>
<td></td>
<td>Colonized-controls</td>
<td>0.85 [0.68 – 1.0]</td>
<td>0.41</td>
<td>93%</td>
<td>79%</td>
<td>81%</td>
<td>92%</td>
</tr>
<tr>
<td>CPIS</td>
<td>All controls</td>
<td>0.89 [0.80 – 0.99]</td>
<td>0.45</td>
<td>87%</td>
<td>86%</td>
<td>72%</td>
<td>92%</td>
</tr>
<tr>
<td>(logistic regression)</td>
<td>All controls</td>
<td>0.95 [0.88 – 1.0]</td>
<td>0.38</td>
<td>94%</td>
<td>86%</td>
<td>81%</td>
<td>92%</td>
</tr>
<tr>
<td>CPIS + eNose</td>
<td>All controls</td>
<td>0.76 [0.56 – 0.96]</td>
<td>0.03</td>
<td>89%</td>
<td>75%</td>
<td>89%</td>
<td>75%</td>
</tr>
</tbody>
</table>

Discussion

The present findings suggest that volatile metabolic fingerprinting of TA fluid might discriminate patients with VAP from patients without VAP. Confirmation of these findings in a larger cohort of patients is needed. The diagnostic accuracy was not influenced by the presence or absence of airway colonization. The predicted probability increased towards the day of VAP diagnosis. These data suggest that volatile markers in airway samples can facilitate the diagnostic procedures for VAP before the availability of microbiologic results and warrant further clinical development of this technology.

In the present study, we focused on the diagnostic accuracy of headspace analysis of airways samples with an eNose for the consensus definition of VAP [21]. Bacteria have a distinct metabolism, part of which results in the production of bacteria-specific volatile organic compounds that may be used for diagnosis of the presence or absence of specific strains [13]. Indeed, it has been reported that an eNose can discriminate between growth of Gram–positive bacteria and Gram–negative bacteria, and no bacterial growth in BAL fluid [19]. The number of patients with Gram–positive VAP in our cohort was too small to confirm this. We found that the discrimination between VAP and no VAP with electronic nose analysis was not influenced by bacterial colonization and we did not find any correlation with bacterial growth. Thus our results cannot be fully explained by bacterial
growth alone. Several other factors should be taken into consideration. For example, inflammation and bacterial ecology may modulate bacterial metabolism [33]. Therefore we may not detect bacterial presence per se, but the interaction with the host response.

Importantly, the metabolite fingerprint improved classification of VAP on top of the CPIS. This indicates that VOC detection may be complementary to clinical symptoms for the diagnosis of VAP. Previous studies correlated VOC–profiles to clinical, radiological or microbiological features of VAP alone [18, 20, 34]. These features may not be sufficiently representative for the disease entity VAP while adequate phenotyping of the included patients, thus using a well–defined group of cases, is highly important for teaching a diagnostic algorithm [35]. Therefore, the present study extends the previously observed correlation between VOC–profiles and CPIS, radiology and microbiology and leads to a more confident statement that the observed alterations in volatile metabolite profiles may be due to pneumonia.

The longitudinal data analysis of the period preceding diagnosis showed that the slope of the eNose signal was steeper in patients that were to develop VAP. This slope could be used to discriminate between patients with and without VAP with moderate accuracy. This may imply that the eNose signal can be used to monitor disease progression in an at-risk population.

The strengths of ours study seem to be adequate phenotyping, the combination of the eNose signal with the CPIS and longitudinal analysis. Nevertheless, several limitations should be noted. Firstly, all analyses were performed in a highly selected cohort of patients. It cannot be excluded that this selection promoted the strength of the discriminative signal between VAP and controls by electronic nose fingerprints. Therefore, the present work does not provide data on the diagnostic accuracy in all patients with suspicion on VAP. Generalization is further limited by the modest amount of patients with VAP that were included. We cannot exclude that the observed differences between patients with and without VAP may be due to other causes than the development of VAP. Secondly, tracheal aspirates can only be obtained when sufficient secretions are
present in the trachea. Therefore, we did not have airway samples from every subject at all time points. This may have led to sampling bias, which can influence longitudinal analyses. Finally, we could not identify the VOCs that differentiate between VAP and controls. Although we performed gas chromatography and mass spectrometry, we had difficulties with obtaining a reliable signal due to viscous matrix of the tracheal aspirates. This limits the translation of our findings to the development of new, VAP specific sensor arrays.

While volatile biological markers of airway samples promise to be helpful in distinguishing patients who develop VAP from those who do not develop VAP, the exact role of eNoses in clinical practice is yet uncertain. Even though previous studies suggest a good correlation between exhaled metabolic profiles, called breath–prints, and clinical symptoms or bacteriological growth [18, 20, 36], all these results require robust confirmation in future clinical trials in intubated and ventilated patients. Then it should be shown that eNose signals are complementary to those clinical parameters summarized in the CPIS. And then we can start performing studies in which we try to determine the clinical usefulness of a strategy that uses eNose signals, e.g., to see if use of eNoses leads to more timely use of antibiotics in patients who do develop VAP, or prevents overuse of antibiotics in patients who do not develop VAP.

In conclusion, volatile biomarkers can be complementary to clinical disease markers for improving the diagnosis of VAP. Diagnostic accuracy did not depend on colonization of the airways. Our data warrant the next step in establishing the diagnostic value of volatile organic compounds in VAP by performing longitudinal exhaled breath analysis in a larger population.

References


Chapter 9.

Volatile Metabolites in Exhaled Breath of Ventilated Patients during Bacterial Colonization and Pneumonia

*Not published*

Lieuwe DJ Bos, Marcus J Schultz & Peter J Sterk
Abstract

Exhaled breath of patients with pneumonia contains volatile molecules that are produced by invading pathogens and the host, which alone or in combination could be used as a marker for pneumonia. The aim of this study was to identify volatile molecules in exhaled breath of intubated and ventilated patients at the intensive care unit (ICU) that are associated with pneumonia, colonization of the respiratory tract and markers of inflammation.

Breath from intubated and ventilated ICU patients was analysed using gas chromatography and mass spectrometry during the first day of admission in a prospective design. Pneumonia was defined by clinical, radiological and microbiological criteria. Tracheal aspirates were cultured to determine the presence of bacteria in the lower airways. Levels of inflammatory markers were measured in plasma.

We analysed exhaled breath of 93 patients. 12 patients had probable/proven pneumonia and 25 patients had positive sputum cultures. The concentration of 1-propanol was significantly lower in exhaled breath of patients with probable/proven pneumonia than in patients without pneumonia and those with negative aspirate cultures (p-value: $10^{-4.1}$, fold change: -4.6). Concentrations of heptanal and 1-pentanol were significantly lower in patients with positive tracheal aspirate cultures as compared to those without (p-value: $10^{-3.4}$, fold change: -1.3, for heptanal and p-value: $10^{-3.4}$, fold change: -0.6 for 1-pentanol, respectively). Multiple individual VOCs correlated with circulatory markers of inflammation, but most profoundly with interferon-gamma.

Our data indicate that ventilated patients with pneumonia have a decreased concentration of 1-propanol in breath. Apparently, such decrease is not directly associated with positive sputum cultures or markers of systemic inflammation.
Introduction

Severe community- and hospital-acquired pneumonia (CAP and HAP), with admission to the intensive care unit (ICU) and need for mechanical ventilation, represents a major clinical problem associated with a high mortality [1, 2]. Currently, the diagnosis of pneumonia is based on clinical, radiological and microbiological criteria, but the spectrum of practice is heterogeneous [2] and might benefit from objective assessment by means of biological markers. Little is known about the diagnostic value of biological markers for the diagnosis of pneumonia [3, 4], especially in patients with severe pneumonia that are admitted to the ICU. Biomarkers for the diagnosis of pneumonia may detect the (pulmonary) inflammatory response or the presence of bacteria in the lung.

Bacterial metabolism can change the composition of volatile organic compounds (VOCs) in the exhaled breath, which may be used for sensitive detection of bacterial presence or the specific identification of pathogenic strains [5, 6]. Leukocytes are also known to produce VOCs [7] and since polymononuclear cells are a hallmark of pneumonia these could also contribute to exhaled VOCs [8]. VOCs may very well have biological functions. There is within-species and inter-species communication by the production of VOCs [9-11] and the bacteria may also use volatile molecules to interact with the host and vice versa [11].

The aim of this study was to identify volatile molecules in the breath of intubated and ventilated ICU-patients that are associated with pneumonia, colonization of the respiratory tract and markers of inflammation. We hypothesized that VOCs are present in different concentrations in (1) patients with confirmed pneumonia compared to patients without any signs of pneumonia and (2) patients with positive and negative cultures of tracheal secretions. Furthermore, we hypothesized that VOCs are correlated with specific systemic markers of inflammation (interleukin (IL)-1b, IL-6, IL-8, IL-10, IL-13, tumor necrosis factor (TNF)-alpha, interferon-gamma (IFN-g) and granulocyte-macrophage colony-stimulating factor (GM-CSF)). To that end, breath from newly admitted intubated and ventilated ICU patients was analysed using gas-chromatography and mass-spectrometry (GC-MS).
Methods

Ethical approval and informed consent

The institutional review board of the Academic Medical Center, Amsterdam, The Netherlands, decided that the study did not fulfil the criteria for medical research that requires ethics review according to the Dutch 'Law on medical research', because of the non-invasiveness and absence of burden of examining exhaled air (IRB: 10.17.0729). Therefore, it was judged by the institutional review board that exhaled breath can be analyzed without informed consent of the patient. The study was registered at the Dutch Trial Register (NTR 2750, www.trialregister.nl). Waste material from routine laboratory measurements in blood was collected, centrifuged at 1500g at room temperature for 15min and stored within 4 hours after blood draw at -80º in all patients that were admitted to the ICU. Plasma storage was done within a multi-centre bio-bank study [Molecular Diagnosis and Risk Assessment of Sepsis (MARS): NCT01905033, www.clinicaltrials.gov]. An opt-out consent procedure was approved by the institutional review board.

Design, subjects and setting

In this prospective single center cross-sectional cohort study, all subsequent patients were included within 24 hours of ICU admission if intubated and ventilated, between December 2011 and November 2013. Previous ICU admission and cardiopulmonary surgery were reasons for exclusion. Patients were categorized into two groups: pneumonia and without pneumonia. Exhaled air and peripheral blood samples were taken within 24 hours after ICU-admission.

Clinical diagnosis of Pneumonia

A team of trained clinical research fellows prospectively scored the presence of pneumonia based on adapted Center for Disease Control–criteria and a post–hoc likelihood of infection was scored (none, possible, probable or proven; see figure E1 and table E2 in the supplementary material). All assessors had attended meetings in which clinical case vignettes were discussed and had at least 6 months of work experience [12].
Exhaled breath analysis

Exhaled breath was sampled and analyzed by standardized methodology that was previously published [13]. In short, breath was collected through a disposable side-stream connection for 10 minutes and VOCs were stored on a sorbent tube. These tubes were analyzed by means of GC-MS resulting in multiple ion-fragments per VOC. The abundance of ion-fragments within a small window of retention times (+/- 5 seconds) were grouped if they were strongly correlated (correlation coefficient > 0.7) to limit collinearity of the predictor matrix (e.g. to get one intensity per patient per VOC) but still allow for differentiation between co-elutions.

Microbiology

Tracheal aspirates were obtained from all patients as part of standard surveillance cultures (in the setting of selective decontamination of the digestive tract [14]) and sent for semi-quantitative bacterial culture. Cultures were considered positive if a potential respiratory pathogen was “highly present” (around 10^5 CFU) [15].

Host response

The host response was investigated by the measurement of inflammatory mediators (interleukin (IL)-1β, IL-6, IL-8, IL-10, IL-13, tumor necrosis factor (TNF)-alpha, interferon-gamma (IFN-g) and granulocyte-macrophage colony-stimulating factor (GM-CSF)) in plasma using a cytometric bead array (CBA) Flex Set multiplex assay (BD Biosciences, San Jose, CA) in accordance with the manufacturers’ recommendation. These markers represent different functional classes of cytokines [16]: pro-inflammatory mediators: IL-1β and TNF-α; anti-inflammatory mediators: IL-10 and IL-13; angiogenic cytokines: IL-6 and IL-8; chemokines: IL-8; colony stimulating factors: GM-CSF; T-helper 1 response and macrophage activation: IFN-g.

Group allocation

We divided the population into several groups to answer the three research questions. First, we used confirmed pneumonia (probable/proven, see
clinical diagnosis of pneumonia) patients as cases and patients without suspected pneumonia and negative sputum cultures from the tracheal aspirate as controls. Second, we used patients with positive sputum cultures, regardless of their pneumonia status, as cases with colonization and patients with negative sputum cultures as controls. Finally, we looked at the correlation of exhaled VOCs with inflammatory mediators in the whole patient population.

**Statistical analysis**

Differences between the groups were compared using the Mann–Whitney U or Kruskal–Wallis test for continuous variables and chi–square for categorical variables. Data were summarized using the median and 25–75\textsuperscript{th} percentile for skewed variables and with mean and 95\%-confidence interval (CI) for normally distributed variables and with count and percentage for categorical variables. VOC and inflammatory mediator concentrations were 10-log transformed to obtain a normal distribution. All analyses were performed in R statistics using the R–studio interface [17]. P–values below 0.05 were considered significant.

For the VOCs, we calculated p-values (with the Mann-Whitney U test), fold-change and area under the receiver operating characteristics (ROC-AUC) between cases (pneumonia or positive sputum cultures) and controls. Subsequently, the label (case/control) was permutated and these analyses were repeated for 1000 times. The lowest p-value that was obtained in less than 5\% of these random scenarios was calculated and used to identify significantly altered VOCs using the actual label. For correlation with inflammatory markers the p-values were obtained using Spearman’s correlation and the optimal cut-off was set at the highest value for alpha where the false discovery rate was below 5\% (thus on theoretical basis, not on simulations as before). All results were displayed in volcano-plots and significant VOCs were manually identified using library matching (National Institute of Standards and Technology) and the injection of a pure chemical standard.
Table 1: Patient Characteristics

<table>
<thead>
<tr>
<th></th>
<th>Control negative cultures (N=47)</th>
<th>Control; positive cultures (N=13)</th>
<th>Possible pneumonia (N=21)</th>
<th>Probable pneumonia (N=12)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>59</td>
<td>64</td>
<td>63</td>
<td>61</td>
<td>45-72</td>
</tr>
<tr>
<td>Gender</td>
<td>Male</td>
<td>28</td>
<td>8</td>
<td>15</td>
<td>5</td>
</tr>
<tr>
<td>APACHE IV Score</td>
<td>80</td>
<td>76</td>
<td>77</td>
<td>66</td>
<td>59-83</td>
</tr>
<tr>
<td>Admission type</td>
<td>Medical</td>
<td>31</td>
<td>8</td>
<td>20</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>Elective Surgery</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Emergence Surgery</td>
<td>12</td>
<td>5</td>
<td>1</td>
<td>4.8</td>
</tr>
<tr>
<td>Respiratory comorbidities</td>
<td>2</td>
<td>4.3</td>
<td>0</td>
<td>6</td>
<td>28.6</td>
</tr>
<tr>
<td>Malignancy</td>
<td>4</td>
<td>8.5</td>
<td>3</td>
<td>4</td>
<td>19</td>
</tr>
<tr>
<td>Diabetes</td>
<td>4</td>
<td>8.5</td>
<td>3</td>
<td>2</td>
<td>9.5</td>
</tr>
<tr>
<td>Maximal inspiratory pressure (cmH2O)</td>
<td>17</td>
<td>14-22</td>
<td>16</td>
<td>13-17</td>
<td>21</td>
</tr>
<tr>
<td>Positive end expiratory pressure (cmH2O)</td>
<td>5</td>
<td>5-5</td>
<td>5</td>
<td>5-5</td>
<td>8</td>
</tr>
<tr>
<td>Minute ventilation (L/min)</td>
<td>9.6</td>
<td>8.3-11.3</td>
<td>8.9</td>
<td>7.7-10.9</td>
<td>10.5</td>
</tr>
<tr>
<td>PaO2/FiO2 (mmHg/%)</td>
<td>280</td>
<td>243-377</td>
<td>349</td>
<td>294-467</td>
<td>300</td>
</tr>
<tr>
<td>Gram Positive isolation tracheal aspirate</td>
<td>0</td>
<td>0</td>
<td>6</td>
<td>46.2</td>
<td>1</td>
</tr>
<tr>
<td>Gram Negative isolation tracheal aspirate</td>
<td>0</td>
<td>0</td>
<td>8</td>
<td>61.5</td>
<td>2</td>
</tr>
</tbody>
</table>

Continuous variables are expressed as median (25th to 75th percentile). Categorical variables are expressed as number (percentage). Differences between groups (possible/probable pneumonia) are tested using Kruskall Wallis or Chi–square test. APACHE II: Acute Physiology and Chronic Health Evaluation II.
Results

Subjects

300 patients were screened of whom 140 did not meet the inclusion criteria, mostly because they were not mechanically ventilated (figure 1). Of the 160 eligible patients, 59 met exclusion criteria (42 were previously mechanically ventilated and 17 were missed) thus 101 patients could be included. Eight additional patients could not be used because the GC-MS results were too deviant (as defined by >3 SD in principal component analysis) to allow for meaningful statistical analyses. The clinical and microbiological data on these patients are summarised in table 1.

Figure 1: Inclusion chart
Volatile organic compounds

1246 ion-fragments were found in all breath samples. Grouping of these ion-fragments based on retention time and correlation coefficient resulted in the separation of 140 VOCs.

Probable pneumonia vs. control patients with negative tracheal cultures

12 patients were classified as probable/proven pneumonia and 47 as patients without pneumonia and without positive sputum cultures. Figure 2A shows the volcano plot. 1-Propanol was significantly lower in the breath of patients with probable/proven pneumonia than in patients without pneumonia and without positive sputum cultures (p-value: $10^{-4.1}$, fold change: -4.6, AUC-ROC: 0.85). Figure 3 shows the abundance of 1-propanol in patients in all patients, stratified per likelihood of pneumonia.

Patients with positive vs. negative tracheal cultures

25 patients had positive and 68 patients had negative sputum cultures, irrespective of the suspicion of pneumonia. Figure 2B shows the volcano plot. Heptanal and 1-pentanol were significantly lower in patients with positive sputum cultures (p-value: $10^{-3.4}$, fold change: -1.3, AUC-ROC: 0.74 for heptanal and p-value: $10^{-3.4}$, fold change: -0.6, AUC-ROC: 0.74 for 1-pentanol, respectively).

Correlation with markers of systemic inflammation

None of the VOCs passed the threshold for statistical significance for the correlation with IL-1β, IL-6, TNFα and GM-CSF. Table 2 shows the significant correlations between IL-8, IL-10, IL-13, IFN-gamma and individual VOCs.
Figure 2: Vulcano plots

Figure 3: 1-Propanol per group

Figure 3: 1-Propanol per group
## Table 2: Correlation between inflammatory markers and VOCs

<table>
<thead>
<tr>
<th>Inflammatory marker</th>
<th>VOC</th>
<th>Correlation coefficient</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-8</td>
<td>Methyl-cyclopentene</td>
<td>0.44</td>
<td>$10^{-4.6}$</td>
</tr>
<tr>
<td>IL-10</td>
<td>Methyl-cyclopentene</td>
<td>0.46</td>
<td>$10^{-5.1}$</td>
</tr>
<tr>
<td>IL-10</td>
<td>2-Methyl-2-Propanol</td>
<td>0.38</td>
<td>$10^{-3.6}$</td>
</tr>
<tr>
<td>IL-13</td>
<td>Unknown</td>
<td>-0.35</td>
<td>$10^{-3.0}$</td>
</tr>
<tr>
<td>IL-13</td>
<td>Unknown</td>
<td>-0.36</td>
<td>$10^{-3.3}$</td>
</tr>
<tr>
<td>IL-13</td>
<td>à-Pinene</td>
<td>-0.39</td>
<td>$10^{-3.8}$</td>
</tr>
<tr>
<td>IFNg</td>
<td>Cyclohexanone</td>
<td>0.36</td>
<td>$10^{-3.1}$</td>
</tr>
<tr>
<td>IFNg</td>
<td>1-Pentanol</td>
<td>0.68</td>
<td>$10^{-3.6}$</td>
</tr>
<tr>
<td>IFNg</td>
<td>Heptanal</td>
<td>0.31</td>
<td>$10^{-2.5}$</td>
</tr>
<tr>
<td>IFNg</td>
<td>Hexanal</td>
<td>0.35</td>
<td>$10^{-3.1}$</td>
</tr>
<tr>
<td>IFNg</td>
<td>2-Propenoic acid, 2-methyl-, ethyl ester</td>
<td>0.31</td>
<td>$10^{-2.5}$</td>
</tr>
<tr>
<td>IFNg</td>
<td>2-Pentanone, 3-methyl-</td>
<td>0.34</td>
<td>$10^{-2.9}$</td>
</tr>
<tr>
<td>IFNg</td>
<td>Unknown</td>
<td>0.34</td>
<td>$10^{-3.0}$</td>
</tr>
<tr>
<td>IFNg</td>
<td>Unknown</td>
<td>0.32</td>
<td>$10^{-2.5}$</td>
</tr>
<tr>
<td>IFNg</td>
<td>C9-alkene</td>
<td>0.32</td>
<td>$10^{-2.6}$</td>
</tr>
<tr>
<td>IFNg</td>
<td>2-Butanol</td>
<td>0.37</td>
<td>$10^{-3.4}$</td>
</tr>
<tr>
<td>IFNg</td>
<td>Unknown cyclic compound</td>
<td>0.34</td>
<td>$10^{-2.9}$</td>
</tr>
<tr>
<td>IFNg</td>
<td>Unknown</td>
<td>0.31</td>
<td>$10^{-2.4}$</td>
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<td>IFNg</td>
<td>Unknown</td>
<td>0.31</td>
<td>$10^{-2.5}$</td>
</tr>
<tr>
<td>IFNg</td>
<td>Unknown</td>
<td>0.32</td>
<td>$10^{-2.6}$</td>
</tr>
<tr>
<td>IFNg</td>
<td>Benzene, (1-methylethyl)-</td>
<td>0.31</td>
<td>$10^{-2.4}$</td>
</tr>
<tr>
<td>IFNg</td>
<td>C9-alkene</td>
<td>0.32</td>
<td>$10^{-2.6}$</td>
</tr>
</tbody>
</table>
Discussion

The data presented in this study indicate that pneumonia is associated with a decrease in the concentration of 1-propanol in breath of ventilated intensive care unit patients. A positive culture of tracheal aspirate with a respiratory pathogen was associated with a decrease in 1-pentanol and heptanal. Inflammatory mediators in plasma were correlated with multiple VOCs. The most profound correlation was found for interferon-gamma. These results warrant validation of the diagnostic value of VOCs for pneumonia in clinically relevant patients populations on the intensive care unit. The association between the presence of pneumonia and a decreased concentration of 1-propanol represents a novel observation. It has previously been suggested that breath analysis could be used to diagnose pneumonia in ventilated patients [18, 19], but the VOCs that differentiate have never been identified before. 1-propanol has been linked to bacterial metabolism in vitro [5]. In contrast to our results, in vitro studies observed higher concentrations of 1-propanol during bacterial growth [5]. This suggests that it may not be bacterial metabolism that is detected by this breath test, but another feature of pneumonia reflecting host-pathogen interaction. The latter hypothesis is further supported by the fact that the breath concentration of 1-propanol was not different between patients with and without positive sputum cultures.

Positive sputum cultures were associated with lower concentrations of 1-pentanol and heptanal. In line with the observation in the previous paragraph, 1-pentanol is found in higher concentration during bacterial growth in vitro. However, it should be noted that bacterial metabolism is highly dependent on the availability of specific substrates [5]. Our data suggest that bacteria consume instead of produce 1-pentanol during growth in vivo. Alternatively, positive sputum cultures essentially reflect overgrowth of one specific bacterial strain, which inhibits the metabolism of the "normal" lung microbiome and thereby decreases the concentration of specific bacterial metabolites.

Heptanal is produced during peroxidation of n-9 unsaturated fatty acids in humans [20], which suggests that oxidative stress is decreased during bacterial growth. However, an increase in lipid peroxidation may have
been expected during bacterial colonization as oxidative stress is part of the innate immune response [21]. We could speculate that patients with a decreased oxidative response to pathogens have increased bacterial growth and therefore a decrease in heptanal was observed, although we have no data to support that hypothesis. Moreover, the positive correlation between heptanal and interferon gamma supports the biological plausibility of our findings, suggesting that inflammation (increased T-helper 1 response and macrophage activation) is associated with lipid peroxidation. An alternative explanation may be that the amount of n-9 unsaturated fatty acids is lower in patients with positive sputum cultures. However, to our knowledge there is currently no evidence that links bacterial growth to decreased concentrations of n-9 unsaturated fatty acids. Thus the association between positive bacterial cultures and decreased heptanal concentrations remains to be explained.

Several VOCs were correlated with markers of inflammation. For example, methyl-cyclopentene positively correlated with IL-8 and IL-10. IL-8 is a chemo-attractant and leads to the accumulation of inflammatory cells on site whereas IL-10 is anti-inflammatory. A pro-inflammatory response is typically followed by an anti-inflammatory counter reaction and therefore the two contra regulatory processes may be correlated in vivo. The biochemical link between methyl-cyclopentene and inflammation is unclear and has not been described in literature. That also holds for all other VOCs that are linked to inflammation in this study. Pre-clinical studies are mandatory to clarify the relationship between inflammation and the observed individual VOCs in this and previous studies [22].

We believe this study has several strengths. We used an unbiased approach for the identification of VOCs that are associated with pneumonia. Furthermore, we did not limit the analysis to the clinical syndrome consensus diagnosis of pneumonia, but also used more objective biological measures such as bacterial growth and inflammatory response. However, several limitations should also be noted. First, the included groups are easily differentiated based on other characteristics and there is no clinical need for a biological marker in this patient population. Second, even though we took all statistical measures to suppress false positive results, the sample size was limited. This may hamper the generalization
of the results and awaits independent validation. Furthermore, the limited sample size prevented us from analyses between different individual bacterial strains. The latter is obviously important in view of clinical usage of breath tests in pneumonia. Third, systemic inflammation was assessed but inflammation within the pulmonary compartment may be of more interest in pneumonia [8, 23, 24]. Finally, univariate analysis was performed to identify potential biological markers for pneumonia, bacterial growth and inflammation but composite markers were not investigated. It is well established that markers that show good univariate classification are frequently outperformed by combinations of markers that show moderate univariate classification [25]. Because of the high dimensionality of the predictor matrix, the limited number of cases and the absence of an external validation cohort multi-variate analysis was of limited added value in this proof-of-concept study, but should definitely be considered in any larger validation cohort, especially if the classification paradigm is the main objective of the study.

We identified several potential breath markers for pneumonia and positive sputum cultures. However, as stated before, our results merely provide a first identification of the VOCs that could be of diagnostic value in pneumonia. Validation would require the use of extensive diagnostic procedures to obtain a gold-standard reference diagnosis for pneumonia in a population that has a high clinical suspicion of pneumonia. Possibly, the most clinically relevant patient population is those suspected of ventilator-associated pneumonia. Breath analysis could thereby assist to determine which patient should and should not be treated with antibiotics, very similar to cytokine analysis of BAL-fluid [26, 27], but without the need for an invasive diagnostic procedure.

Conclusion

Our data suggest that pneumonia in ventilated patients is associated with a decreased concentration of 1-propanol in breath. Lower concentrations of 1-pentanol and heptanal in breath were associated with positive sputum culture of a respiratory pathogen. Inflammatory mediators in plasma were correlated with multiple individual VOCs. The most profound correlation was found for interferon-gamma. These results warrant validation of the
diagnostic value of VOCs for pneumonia in clinically relevant patients populations on the intensive care unit.

References

12. Klein Klouwenberg PMC, Ong DSY, Bos LDJ, de Beer FM, van
Part IV

Discussion
Chapter 10.

General discussion

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Lieuwe DJ Bos
Introduction

Ventilated patients in the intensive care unit (ICU) can present with, or develop pulmonary injury (acute respiratory distress syndrome; ARDS) and/or pulmonary infection (pneumonia) [1-3]. ARDS and pneumonia are both known to be associated with an inflammatory response [4, 5]. In animal models, a response can be observed in the systemic and pulmonary compartment. In samples from patients, local markers of the innate immune response seem to allow for better discrimination with a clinically relevant critically ill control group than systemic markers [6-8]. Thus when investigating biological markers of ARDS or pneumonia sampling from the pulmonary compartment should be preferred above blood sampling. However, it remains difficult to obtain a sample from the lungs with broncho-alveolar lavage, as this can introduce burden and risk and certainly cannot be performed very frequently. Therefore, the following central postulate was at the basis of this thesis: there is a need for a non-invasive tool that can assess pulmonary molecular markers in order to predict and diagnose ARDS and pneumonia in intubated and ventilated ICU-patients [9-15].

Besides several abundant molecules such as nitrogen, oxygen and carbon dioxide, breath also contains volatile organic compounds (VOCs) at very low concentrations (parts-per-billion). These molecules represent (fragments of) metabolic products and can be of systemic origin and transported to the lung via the circulation or can be produced locally in the lung [16]. Several mechanisms could lead to the formation of VOCs. Some are produced with ketone formation and other processes in central metabolism [17]. Oxidation of fatty acids may result in alkanes, alkenes and aldehydes [18]. In vitro experiments suggest that leukocytes can produce VOCs directly [19]. Nitrogen and sulfide containing organic molecules are normally eliminated via the liver and the kidney and their concentration may rise in the breath if the clearing function of these organs is limited [20, 21]. Micro-organisms also contribute to the exhaled VOCs. Gut bacteria produce volatile compounds with fermentation. More relevant for pneumonia, bacteria in the lung may also produce VOCs [22] either or not affected by interaction with the host. Finally, many compounds in the breath may be derived from the environment; they were previously inhaled, represent metabolites derived from food or drinks or reflect...
volatile drugs or drug metabolites [23, 24].

VOCs can be detected in various manners. In this thesis we focused on two distinct approaches: a) gas-chromatography and mass-spectrometry for the separation, identification and quantification of potential volatile biomarkers and b) electronic nose analysis for the recognition of complex VOC mixtures by capturing “molecular fingerprints”, which present patterns for ARDS or pneumonia and may be used for empirical classification. To allow for breath analysis in ventilated ICU-patients, a new, simplified breath collection system needed to be developed. The latter technology was used to identify volatile biomarkers in breath that may be useful for early recognition and diagnosis of ARDS and pneumonia. We also investigated the accuracy of a commercially available electronic nose for the empirical diagnosis of ARDS and ventilator-associated pneumonia (VAP). The results on ARDS and pneumonia were described in two separate parts of the thesis and will be discussed separately hereafter (part I on page 213, part II on page 225), followed by the general implications for breath analysis in ventilated patients (part III on page 231).

**Part I: Acute respiratory distress syndrome**

**Summary**

A new, simplified breath collection method was developed that allows for safe and quick collection of breath in mechanically ventilated patients (chapter 3). A side-stream flow allows for the trapping of VOCs onto sorbent material that can be used for GC-MS analysis. Alternatively, direct analysis with an electronic nose can be performed (chapter 4). The method provided reproducible results. Several VOCs were identified that emanated from the ventilator and tubing in high concentrations. These VOCs could *a priori* be excluded as potential biomarkers. In two animal experiments (chapter 5) in which we administrated lipopolysaccharide (LPS) intravenously or intra-tracheally we found that VOCs in the breath of rats change with the development of pulmonary inflammation. Several VOCs overlapped between the two animal models for lung injury and may therefore be considered candidate markers for an inflammatory response. Interestingly, hexanal, pentadecane and 6,10-
dimethyl-5,9-undecadien-2-one were found in lower concentrations after LPS instillation. This was unanticipated as these VOCs are typically associated with oxidative stress, potentially inducing an increase after LPS administration. In chapter 4, we described that breath analysis with a commercially available electronic nose can be used to discriminate patients with ARDS from patients without ARDS with moderate accuracy. The diagnostic accuracy increased with increasing severity of ARDS. However, we had to conclude that as yet the currently used eNose technology is not applicable in clinical practice, because the test accuracy was too low and the sensors showed marked drift over the inclusion period. In chapter 6 we used a similar study design as in the previous chapter but applied gas-chromatography and mass-spectrometry instead of electronic nose analysis as the index test. We found that the combined signal of three metabolites (octane, 3-methyl heptane and acetaldehyde) in the breath could be used to discriminate patients with ARDS from patients without ARDS with good accuracy. This accuracy was maintained in a temporal external validation cohort, which limits the chances that discrimination resulted from a type-II error. Furthermore, the exhaled breath signal improved diagnostic accuracy on top of a clinical prediction score (the lung injury prediction score; LIPS).

Taken together, these data demonstrate that specific VOCs in the exhaled breath can be used to discriminate patients with ARDS from patients without ARDS, suggesting that exhaled breath analysis may be used for the diagnosis of ARDS.

**Bayesian intermezzo**

This section serves the purpose to explain the elaborate description of biological mechanisms that possibly lead to the formation of VOCs on the following pages. The research described in this thesis is performed with an “omics” perspective, meaning that no target markers were selected *a-priori*. That approach is frequently criticized with arguments such as “the fishing expedition”, referring to the chances on type I errors with this kind of research. Therefore, it is highly important for the community performing these studies, to be maximally self-critical. As described in every chapter, we have to
limit the chances of false-discovery (thus type I errors) by means of statistical procedures such as cross-validation and confirmation studies in independent cohorts (external validation).

In this intermezzo I want to carefully make the point that this may not be sufficient. All studies described in this thesis and most studies published in literature use variations of Fisher’s statistics and the inevitable p-values. Although most chapters focus on effect sizes and not statistical significance per se, the a priori (before experiment) probability of the hypothesis being true were never taken into account. However, with an a-priori 19 to 1 odds against the hypothesis (the long shot) even a p-value of 0.01 gives a post-test probability of only 30% on a “real” effect. An a-priori odds of 1:1 (coin toss) gives a post-test probability of 89% using the same p-value. Only a good bet (9:1 odds) gives a 99% post-test probability with p-value of 0.01 [25]. Why is this important? Because we make no assumptions on pre-test probability in “omics” research and typically apply the same cut-off to identify significantly associated markers, our conclusions about post-test probability ought to be heavily influenced by biological plausibility [26]. For example, if breath analysis reveals two compounds that are similar in discrimination for ARDS (same area under the receiver operating characteristics curve) and reject the NULL hypothesis with the same confidence (similar p-value and confidence interval) but one compound is an aromatic, fluorated compound whereas the other is a C8 alkane (octane) the post-test probabilities would differ dramatically. As aromatic fluorated compounds are not known to be produced by human metabolism the pre-test probability is close to 0 and even a very small p-value will increase that probability to not more than 10-30%. Alternatively, octane is known to be produced by peroxidation of fatty acids (see following section) and there is literature that involves that process in the pathogenesis of ARDS. Therefore, the pre-test probability was good for that compound and this biological marker should be favored for additional experiments. Importantly, the systems biologists within our community will certainly criticize these clausules. They state that if a priori probability would always be taken into account, radically new things would never be discovered.
That reaction is valid to a certain extend. However their defence is also partly based on two errors in the estimation of probability: availability (overestimating the frequency of vivid or easily recalled events) and probability transformations (small probabilities are overweighted and large probabilities are underweighted) [27]. In fact, this Bayesian-like approach could allow for radical discoveries. If something has a very low pre-test probability but is shown in several replications within different populations, the likelihood for a true positive finding steadily increases. In other words, to find the middle ground before continuing, this intermezzo describes a moderate view on things. Let us not discard hypotheses directly that have a very high a-priori probability if we fail to reject the NULL hypothesis, but let us also not claim things if we find a low p-value, without careful consideration of proper replication and biological plausibility.

**Biological mechanisms**

*Animal versus patient studies*

In the present thesis, the volatile organic compounds that are associated with ARDS were investigated in a translational setting. However, the VOCs that were found in the animal model were different from those found in the human studies. As mentioned in the summary, the decreased concentrations of hexanal, pentadecane and 6,10–dimethyl–5,9–undecadien–2–one in the animal study were peculiar as the opposite would be expected with increased oxidative stress. In the interpretation of these findings, the mild nature of the lung injury found in our animal model should be taken into account. Although we did observe marked inflammation in the animals that received LPS, markers of permeability were not different between the two groups. Additionally, we analyzed the changes in breath VOCs after LPS administration in the animal studies but in the human study we took a single sample in established ARDS. Therefore, direct comparison of these findings is difficult. In hindsight, it could be argued that an animal model with severe lung injury could have provided more information in this stage. We could conclude from the animal model that LPS administration results in decreased exhaled concentrations of hexanal, pentadecane and
6,10-dimethyl-5,9-undecadien-2-one. However, in patients these VOCs may very well not be the best markers for discrimination between two patient populations of which one developed (diagnosis) or will develop (prediction) ARDS. In that sense, the animal study addressed the issue of monitoring whereas the human studies focused more on diagnosis.

**Lipid peroxidation**

Octane, a C₈ unbranched hydrocarbon, discriminated best between patients with ARDS and without ARDS. Octane was higher in patients with ARDS. Lipid peroxidation of oleic acid (C₁₈:₁ n-9) is the most likely mechanism by which octane is produced in the body. As shown in figure 1, that mechanism is highly predictable based on the chemical structure of the fatty acid. Besides octane, several other hydrocarbons (heptane, 1-nonene and 1-decene) would result from peroxidation of oleic acid [28]. Other relatively abundant fatty acids give rise to similar VOCs, that could also be detectable in the breath of patients (e.g. hexane and 1-octane as a result of n-7 unsaturated fatty acid peroxidation and pentane from peroxidation of n-6 unsaturated fatty acids, such as linoleic acid). Two pathophysiological mechanisms can result in the observed increased concentration of octane in the breath of patients with ARDS. The first postulate is that ARDS increases oxidative stress, which increases the likelihood that fatty acids are peroxidized and thereby leading to all alkanes (and other peroxidation products) being increased in ARDS. The second, alternative, postulate is that the concentration of oleic acid is increased in ARDS and, irrespective of the peroxidative flux, thereby increasing the concentration of octane. A third possible explanation might be decreased degradation of alkanes. Humans cannot metabolize alkanes, but some micro-organisms can [29]. However, these enzymes are not specific for octane and typically cover a spectrum of C₅-C₁₀ [29]. It should be stated that these hypotheses are not completely mutually exclusive. We were able to test these hypotheses by a combination of post-hoc analysis and literature review. If the first or third scenario were true, other alkanes would also be different between ARDS and controls. Therefore, a post-hoc analysis was performed for hexane and pentane in the study that was described in chapter 6. Figure 2 shows that the concentrations of alkanes not produced by oleic acid peroxidation were not significantly increased in ARDS, thus rejecting the first hypothesis.
Figure 1: Peroxidation of fatty acids to alkanes and alkene

- Fatty acid (C18 n-9) Oleic acid
- 10-Hydroperoxide
- 8-octadecenoic acid
- Alkoxy radical

\[ CH_2(\text{CH}_2)_{7}\text{CH}=\text{CH}(\text{CH}_2)_{7}\text{COOH} \]

\[ CH_2(\text{CH}_2)_{7}\text{CH}=\text{CH}(\text{CH}_2)_{6}\text{COOH} \]

\[ CH_2(\text{CH}_2)_{7}\text{CH}=\text{CH}(\text{CH}_2)_{6}\text{COOH} \]

\[ CH_2(\text{CH}_2)_{7}\text{CH}=\text{CH}(\text{CH}_2)_{6}\text{COOH} \]

\[ CH_2(\text{CH}_2)_{7}\text{CH}=\text{CH}(\text{CH}_2)_{6}\text{COOH} \]

\[ CH_2(\text{CH}_2)_{7}\text{CH}=\text{CH}(\text{CH}_2)_{6}\text{COOH} \]

7-Hydroperoxide

8-Hydroperoxide

9-Hydroperoxide

10-Hydroperoxide

Heptane
Octane
1-Nonene
1-Decene

Figure 2: Peroxidation products in ARDS

The upper row displays the concentration of alkanes and alkenes that are produced during the peroxidation of oleic acid. The bottom row for products of peroxidation of n-7 and n-6 unsaturated fatty acids. The concentration is given in pg/L.

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The alternative scenario would result in increased concentrations of heptane, 1-nonene and 1-decene in ARDS. Indeed, the concentration of these three VOCs was increased in patients that suffered from ARDS, albeit not as markedly as for octane. These results are in line with the hypothesis that the high exhaled octane concentration that we observed in ARDS is most likely explained by an increased concentration of oleic acid.

**Oleic acid**

A review of the literature reveals that an increased plasma concentration of oleic acid (also called oleate and C18:1 n-9 in the literature) can be found in ARDS, even before its clinical manifestation [30, 31]. Actually, that was the motivation for testing lisofylline, a reducer of free fatty acids [31, 32], as a pharmacological treatment in patients with ARDS [33]. Unfortunately, the latter study showed no survival benefit for the intervention group [33]. As stated in the accompanying editorial [34], that might not be unexpected as the intervention was given 36 hours after the patient met the consensus criteria while no animal study showed benefit when administered more than 4 hours after the initial insult [35]. Lisofylline did also not decrease the plasma concentration of a range of fatty acids, including oleic acid [33]. These findings imply that if oleic acid is involved in the pathogenesis of ARDS it is probably in the early phases of disease.

In experimental studies, intravenous administration of oleic acid is a well-known model for lung injury that shows several pathological signs of human ARDS, such as hyaline membranes [36]. However, the dose that is injected is several orders higher than the natural occurring concentrations and mimics the situation of a fat embolism, a very rare cause of ARDS in humans. One paper reports the effects of oleic acid in plasma concentrations that can be observed in patients at risk for ARDS on type I alveolar cells [37]. Oleic acid inhibits Na-channels and Na/K/ATPase in vitro, which results in less fluid clearance from the lungs and could contribute to the exudative phase of ARDS. These results were confirmed in an ex vivo mouse lung [38]. Specific inhibition of Na/K/ATPase by ouabain also resulted in alveolar permeability, although less pronounced than with oleic acid injection. Thus, there is probably a strong role for Na/K/
ATPase in the mechanism by which oleic acid induced pulmonary edema, but additional mechanisms could contribute. Leukotriene B4 (LTB4) and prostaglandin E2 (PGE2) are suggested as a down-stream coupling with inflammation and thereby to injury [38]. Indeed, LTB4 and PGE2 were found to be elevated in trauma patients that were to develop ARDS [39]. However, the mechanism by which PGE2 is up-regulated are unclear as oleic acid does not increase production directly through arachidonic acid [40] but rather via production in the lung itself [41]. Taken together, there is strong evidence for a contribution of oleic acid to the development of ARDS, at least in animal models of lung injury and possibly in human ARDS. Together with the biochemical mechanism that links octane to oleic acid, this may imply that we are able to non-invasively measure a very early pathophysiological mechanism in the development of ARDS.

Several steps have to be taken before breath octane may be used as a clinical predictor for ARDS. The implications for clinical trials will be discussed in the next section. With the currently available data from the study that is described in chapter 6, we can already try to formulate basic assumptions for future studies. In the context of the concurrent MARS (Molecular Diagnosis and Risk Stratification of Sepsis) study, aimed to find biological markers of sepsis and organ failure in ICU-patients, we had cytokine levels in plasma and mini-BAL in ventilated ICU-patients (n=93 for plasma and n=13 for mini-BAL) to our disposal. Figure 3 shows the correlation between several peroxidation products of oleic acid (octane, heptane and 1-nonene) and products of other fatty acids (hexane and pentane) with plasma and pulmonary markers of inflammation. None of the breath alkanes show a correlation with systemic inflammation. Pulmonary inflammation is correlated with several breath alkanes. The products of oleic acid peroxidation show a marked inverse correlation with IL-6 and a positive correlation with IL-10, which suggests that oleic acid peroxidation is associated with anti-inflammatory processes.
Figure 3: Correlation between alkanes/alkenes and inflammatory markers

*Left:* correlation of breath alkanes with markers of inflammation measured in plasma. *Right:* correlation of breath alkanes with markers of inflammation measured in mini-broncho-alveolar lavage. The x-axis shows alkanes and alkenes as peroxidation products from different fatty acids. The y-axis shows inflammatory markers. Correlation coefficients are indicated by a number and colour (blue is negative, red is positive).

The other alkanes also show negative associations with other pro-inflammatory markers, primarily with IL-8. Although these pre-liminary results do not provide us with a mechanistic coupling between fatty acid peroxidation and inflammation, we can safely conclude that there is no association with systemic inflammatory response. Therefore further efforts should focus on inflammation in the lung. Additional measurements in these samples will give us a profile of fatty acids in the plasma and in the lung. If the correlation between absolute oleic acid concentration and breath octane concentration that we postulated on biochemical grounds can be confirmed, we can more confidently state that octane is indeed a surrogate for oleic acid and not for lipid peroxidation *per se*.

**Future clinical implications**

In chapter 6 we showed that exhaled breath analysis provides information on the presence or absence of ARDS and this is complementary to pre-test risk assessment by the lung injury prediction score (LIPS). This may
not be unexpected as the LIPS consists of clinical risk factors, whereas exhaled breath analysis is based on biochemical alterations that occur during ARDS. Hence, a patient can have a very high a priori risk for disease (LIPS), but in absence of the biochemical presentation we may still conclude the patient probably does not have that specific condition (yet). In line with that, if a patient exhibits the biochemical profile with a negligible risk for disease such patient probably does not have the condition either. This may have implications for targeted therapies (figure 4). Until now, most pharmacological (targeted) interventions have been tested in a patient population that was selected based on clinical characteristics. It should be noted that every single pharmacological trial in patients with established ARDS was negative [42].

Our results further extent existing evidence that the clinical syndrome diagnosis of ARDS is not a good surrogate for specific biochemical alterations. However, as long as we cannot measure biological markers that sufficiently represent the biochemical pathway that the pharmacological agent intervenes with it remains to include the patient population that benefits most in clinical trials. Of note, an intervention that is highly effective in a subpopulation but slightly harmful in most patients will probably result in a negative (non-inferiority) clinical trial when an unselected cohort is included. Additionally, prevention of ARDS has probably more potential than treatment of established ARDS. If the assumption that oleic acid is a central mediator in the pathogenesis of ARDS in some patients will show to be correct and octane is a good marker of oleic acid peroxidation, this could be the first molecular marker that can be measured non-invasively, rapidly and at the bedside.

Pre-clinical research could help to further understand the mechanisms that couple oleic acid peroxidation and octane formation, via Koch’s postulates [43, 44]. Questions about dynamics and the potential of therapeutic intervention need to be addressed to allow for optimal design of clinical studies. First of all, octane formation after infusion of oleic acid has never been confirmed in vivo. If such biochemical mechanism is confirmed, several in vitro and in vivo experiments should be conducted to evaluate the dynamics of that association.
Figure 4: Model for development of ARDS and timing of therapeutic intervention.

Relevant questions are whether oleic acid injection is always followed by alkane formation, what is the delay and can the association be uncoupled in certain conditions (excess super-oxide formation)? Most of these questions can be answered by straightforward experimental studies, with the injection of oleic acid and frequent measurement of exhaled octane concentrations. When we want to separate the contribution of endogenous and the infused oleic acid, the infused fatty acid could be labeled with C13, after which octane originating from that fatty acid can easily be separated by means of gas-chromatography and mass-spectrometry.

The influence of therapeutic interventions could also be measured in these models. Despite the negative results of clinical trials, lisofylline and beta agonists may be re-evaluated. Lisofylline lowers the concentration of free fatty acids and could thereby be used as a targeted therapy in
patients with high exhaled octane concentrations (and thus, if the previous assumptions are confirmed, high oleic acid concentration). If given before alveolar flooding occurs, lowering of the serum oleic acid concentration may prevent inhibition of the Na/K/ATPase and thereby the lung may be able to compensate for increased exudate formation in the lung and stop alveolar flooding. Beta agonist can regulate Na/K/ATPase via MAPK/ERK pathways, without the influencing fatty acid concentrations [45] and could thereby increase alveolar fluid reabsorbance. Despite promising pre-clinical studies, randomized controlled trials in patients with established ARDS did not show benefit of beta agonists [46]. ARDS was also not prevented by beta agonists in a high-risk surgical population [47]. However, the latter trial did show less extra-vascular lung water in the intervention group. The regulation of Na/K/ATPase by beta agonist also makes this result biologically plausible. Before dismissing these two simple, biologically plausible preventive measurements, post-hoc analysis may provide additional evidence if a subgroup does benefit from the intervention. Subsequently, that can be prospectively evaluated in a new clinical trial.

In line with the previous comments on pharmacological prevention of ARDS, biological markers have most potential if used for the prediction of ARDS. Therefore, the diagnostic accuracy we found in chapter 6 should be confirmed in large, multi-center, longitudinal observational study. A cohort of patients at risk for ARDS should be included. Samples should be collected frequently so that the moment of biochemical presentation of ARDS is not missed. Two settings can be anticipated. First, mechanically ventilated patients undergoing major surgery or admitted to the ICU are at high risk to subsequently develop ARDS. These patients could be monitored using the sample methodology that is described in chapter 3. Another patient group that is at high risk are those admitted to the emergency department, with a lung injury prediction score above three. Sample collection is more cumbersome in this population as it currently requires forced exhalation in a sample collection bag; something that is unpleasant for dyspnoeic patients, time consuming and requires a special infrastructure. On page 217 of this discussion other methodologies for the direct analysis of breath are described.
To summarize, patients with clinical risk factors for ARDS could benefit most from preventive interventions, especially if the nature of the “second hit” could be detected with biological markers. Breath analysis of octane may capture oxidative stress and the process of oleic acid damaging the Na/K/ATPase leading to less alveolar fluid clearance and could thereby provide information for targeted treatment.

Part II: Ventilator associated pneumonia

Summary

We, eukaryotes, are biochemically just a differentiated fusion of two prokaryotes [48]. Bacteria are versatile biochemical factories [49]; they can obtain energy from almost any process perceivable [50]. We hypothesized in chapter 7 that the volatile metabolic products of bacteria may be used to identify the presence of any pathogen and of specific strains of pathogens. We systematically reviewed the literature on associations between volatile organic compounds and six of the most common pathogens on the intensive care unit. All six bacteria of interest produce isopentanol, formaldehyde, methyl–mercaptan and trimethyl–amine. The following volatile biomarkers were found for identification of specific strains: isovaleric acid and 2–methyl–butanal for Staphylococcus aureus, 1–undecene, 2,4–dimethyl–1–heptane, 2–butanone, 4–methyl–quinazoline, hydrogen–cyanide and methyl–thiocyanide for Pseudomonas aeruginosa and methanol, pentanol, ethyl–acetate and indole for Escherichia coli.

The major limitation of all included studies is that evaluation of the VOCs was done in vitro without providing the bacteria with a growth medium that partly represents in vivo conditions. Therefore, we evaluated the diagnostic accuracy of VOC analysis for the diagnosis of VAP without using culture media in chapter 8. In this study, tracheal aspirates were collected every three days from ventilated patients without signs of pneumonia. Some of these patients developed VAP and were included as cases. Tracheal aspirates were thawed and analysed by electronic nose and GC-MS. The electronic nose analysis showed that patients with VAP could be discriminated from patients without VAP and that the “VAP signal” already
increased before the moment of clinical diagnosis. However, patients with colonization but without signs of infection were classified as having no VAP in this study. This suggests that the *in vivo* markers of VAP are not related to bacterial presence or growth *per se*. That could mean the VOC signature that was detected by the electronic nose is mostly attributable to host response, or that bacteria produce other VOC profiles during invasive growth. In that study, we also attempted to identify the VOCs that were higher/lower during VAP. However, the GC-MS analyses were frequently unsuccessful due to absorption of water on the Tenax material and the extremely wide range of concentrations of VOCs. Some samples heavily overloaded the mass-spectrometer, whereas others showed only very low intensity peaks. In chapter 9 we further evaluated the association between VOCs, pneumonia and bacterial presence, albeit *in vivo*. In this study, breath analysis was performed following the methodology that was described in chapter 3. Patients were separated in those that had a definite pneumonia at admission and those without any signs of pneumonia. 1-Propanol was found to be significantly lower in the breath of patients with pneumonia than in those without pneumonia. Patients with positive and negative cultures of tracheal secretes were also compared. 1-Pentanol and heptanal were found to be significantly lower in the breath of patients with positive cultures. Finally, VOCs in breath were correlated with markers of inflammation measured in plasma. None of the VOCs passed the threshold for statistical significance for the correlation with IL-1b, IL-6, TNFa and GM-CSF. We observed significant correlated between several VOCs and IL-8, IL-10, IL-13 and interferon-gamma. The positive correlation with interferon gamma was most profound.

Taken together, these data demonstrate that specific VOCs are produced by different bacterial strains, but that these results are not easily translatable into a clinical test for the diagnosis of pneumonia.

**Biological mechanisms**

Our review described in chapter 7 showed that there was sufficient theoretical ground to hypothesize that bacteria contribute to the VOCs in exhaled breath. We did observe changes in the VOC (profile) during pneumonia (chapter 8 & 9), but these could not be contributed to bacterial presence alone. The simplistic view of: bacteria produce VOCs...
there are more bacteria during pneumonia – hence pneumonia can be detected by VOC analysis does not capture the complexity of our results. Therefore, the theoretical framework for pneumonia detection by means of exhaled breath analysis has to be reconsidered.

**In vitro vs. in vivo**

Almost all studies on the association between VOCs and bacteria have been performed *in vitro* with reference strains [22]. Several important factors limit the translation of the results of these studies into the clinical. Firstly, not all studies used the exact same subtype of bacterial species. In one study, phage types of SA influenced headspace volatile organic compounds [51]. Genomic variation between subtypes could result in differences in efficacy of enzymes within a specific metabolic pathway. Certainly, this could hamper the clinical applicability of volatile biomarkers for strain identification. Secondly, the growth medium is the source of building blocks for the produced VOCs and may induce phenotypic alterations in the micro-organisms and it therefore is a confounding factor [51-54]. Without knowledge of the substrates that are available to the pathogen it is uncertain whether an association likely to be observed *in vivo*. In line with that, most studies do report on decreased concentrations of VOCs with bacterial growth, and this could be very relevant in clinical studies as observed in chapter 9. Thirdly, measurements were obtained at different moments in the growth of bacteria. Depletion of metabolites and growth phase (log or stationary) influence headspace metabolites [55-58]. Lastly, patient samples are less well defined than laboratory produced cultures of reference strains and are different in the following aspects: CFU’s, growth phase, host response, viscosity [59], confounding co-morbidities and medications (e.g. antibiotics [60]). Taken together, these limitations suggest that direct translation of *in vitro* results to *in vivo* testing will be difficult. This may explain some of the differences we observed.

**Imperfect reference standard in clinical trials**

Another important difference between most *in vitro* studies and samples collected from patients is that a known amount of a specific bacterial strain is added to a growth medium in the former, whereas an unknown
amount of an unknown bacterial strain is present in the latter. Therefore, the reference test is highly important in studies with clinical materials. We used bacterial culturing methods to first grow and subsequently identify the most common bacteria in the airways. However, it is now widely recognized that there is a wide taxonomy of bacteria present in the lung under normal conditions as well, which cannot be cultured and which may change during disease [61-63]. Therefore, we can hypothesize that our reference standard does not capture the process that we measure with VOC analysis; namely the metabolism of all bacteria in the lung. Several novel, PCR based technologies allow for untargeted analysis of the lung microbiome. This results in a number of copies per bacterial strain (frequently based on the sequence 16S) and can thus be used to evaluate the composition of the bacterial ecology in the lung. Bacterial metagenomic analysis not only replicated and sequences 16S RNA but also detects other bacterial DNA sequences [64]. Therefore, this method can be used to identify whether the bacteria in a sample have the genomic potential to take certain metabolic pathways. This approach was combined with breath analysis in patients with cystic fibrosis. Increased concentrations of 2,3-butanedione were observed in patients with cystic fibrosis and this was linked to an increased number of sequences encoding for proteins that are involved in the biosynthesis of 2,3-butanedione [64]. In combination with data from in vitro experiments and the abundance of different bacterial species (assessed by 16S replication) the authors concluded that Streptococcus species are most likely responsible for the production of 2,3-butanedione. In conclusion, future clinical studies on the association between bacteria and VOCs may benefit from novel sequencing based technologies that allow for a untargeted analysis of the pulmonary microbiome and can quantify the presence of certain sequences encoding for proteins involved in the biosynthesis of these VOCs.

Interplay between bacteria and the host

Bacteria are mostly investigated in mono-culture during in vitro or in vivo experiments. In the clinical situation however, bacteria grow within the ecology of the lung. Within that setting, volatile organic compounds may be used to interact with other bacteria or with the host. For example, several volatile metabolites are known to be involved in quorum sensing [65, 66]; a system that coordinates bacterial gene expression as a consequence of
ecological factors such as population density and substrate availability. Furthermore, bacteria can influence the metabolism of the host for their own advantage [67], which could in theory also result in other volatile metabolic products.

An updated theoretical framework

The VOCs observed in the headspace of mono-cultures of bacteria give an insight into the volatile metabolites that these bacteria can biosynthesize. However, the VOCs that are produced are influenced by growth phase, antibiotic pressure, the availability of substrates and the biochemical pathways that are encoded in the genome of that specific pathogen. Furthermore, VOCs can be used to communicate between bacteria or may be influenced by the host response. Thus, the VOCs measured in the breath of patients with pneumonia could result from bacterial metabolism or host response and are likely influenced by the growth phase, the bacterial ecology and the genome of the pathogen (figure 5). This is the good news for discovering complex mechanisms in host-pathogen interaction, but the bad news for developing unambiguous test in clinical diagnosis and monitoring.

**Figure 5:** Updated theoretical framework (alveolar environment)
Future clinical implications

The theoretical framework described in the previous section has important consequences for future clinical trials. First of all, two explicit separate aims should be differentiated: studies that focus on diagnosis and clinical decision and pathophysiological studies that further explore the associations between VOCs and specific processes within the pulmonary microbiome.

**Diagnostic test for pneumonia**

Studies focused on diagnostic accuracy of a breath test should include only patients with clinical suspicion of the disease (intention to diagnose), where a clinical decision is to be taken (do or do not administer antibiotics) [68]. The reference test should be highly reliable in these patients. For example, if patients with suspected VAP are to be included quantitative cultures of broncho-alveolar lavage fluid could be used as reference standard. This is in stark contrast to studies (such as that described in chapter 9) that use semi-quantitative cultures in a non-selected population as reference standard. These type cultures are far more likely to become positive and the unselected nature of the population increases the probability of (false-)positive cultures, that are clinically irrelevant. For a detailed description of the steps that should be taken with discovery, validation [69] and clinical implication [70] see the section on page 222.

**Association between VOCs and microbiome**

Studies that aim for the discovery of VOCs that represent a specific process within the microbiome of the lung should focus on a complete description of the phenotypic characteristics of the patient that is sampled, with special emphasis on the microbiome that he/she carries around. This includes but is not limited to careful sampling, elaborate analysis of the micro-organisms that are present in the lung and further differentiation of the metabolic pathways that are available to those micro-organisms. The design of such a study could be case-control as the question of diagnostic accuracy is not addressed.
Part III: Exhaled breath analysis

Gas-chromatography and mass-spectrometry

Gas-chromatography and mass-spectrometry is widely recognized as the gold standard for the detection of volatile organic compounds and the discovery of biomarkers in breath [71]. GC-MS can be used to measure a broad range of compounds, for identification of unknown compounds and is semi-quantitative. However, several limitations of the technology should be noted. First, because the technology is mostly not available in the hospital, the sample needs to be stored, for example on an adsorption tube, which will always alter the constitution of the mixture. Second, guiding the sample over one chromatographic column will not separate all VOCs in retention time. As a consequence, low abundance VOCs can be missed and mass spectra are not derived from a single compound, which limits the identification of the compound. Furthermore, choices in absorption material, packing of the column and mass-spectrometer bias the measurement towards certain types of VOCs. For example, in our studies we typically used Tenax as a sorbent. VOCs with a carbon backbone of C4 or shorter do typically not retain very well on Tenax and several very volatile compounds without carbon do not retain at all (e.g. Ammonia). Therefore it may not be surprising that our results typically point to relatively long alkanes as biomarkers. The described limitations do not even include the problems with maintaining a constant retention time per compound, adjustment of the sensitivity of the system and interpretations of the data. Because we lack an alternative for volatile biomarker discovery, gas-chromatography and mass-spectrometry will probably remain the standard for future studies that focus on the identification of volatile metabolites that are associated with certain disease states. The use of more than one GC-MS platform could allow for validation procedures and an extended range of covered metabolites by using partly orthogonal set-ups. Such an approach has been used in clinical labs that specialize in environmental monitoring and also provide excellent, reproducible results in breath research [72-75]. For validation studies, other technologies may be more attractive than GC-MS. These are discussed in the paragraph on “alternative technologies”.

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Electronic nose

As previously stated, electronic nose analysis is attractive from a clinical point of view as it can be available at the bedside and provide results instantly. It does not allow for the measurement of individual VOCs, but uses pattern recognition to capture composite VOC mixtures by cross-reactive sensors, thereby identifying clinically relevant groups of patients. Humans can discriminate approximately 1 trillion olphactory stimuli with only 400 cross-reactive receptors [76, 77], which is the highest resolution of any human sense. For breath analysis, sensitivity towards very low concentrations is equally important as discrimination between different gaseous mixtures. Therefore, a sensor array should meet two important criteria: the sensors should have orthogonal chemical selectivities and should be responsive a wide range of concentrations (low parts per billion to high parts per million).

In chapter 8 we described the discrimination between tracheal aspirates from patients with and without VAP with an electronic nose. The diagnostic accuracy was good in that study. However, breath analysis in patients with and without ARDS provided only moderate discrimination, as shown in chapter 4. Accuracy did increase when we only considered patients with moderate/severe ARDS as cases. Based on the GC-MS study in patients with ARDS (chapter 4) and the literature study of VOCs that could be used for the diagnosis of pneumonia (chapter 7) we can postulate that pneumonia is a disease that is more likely to be discriminated by eNose analysis than ARDS. To explain that, we need to assume that sensor technology will be further developed to the border of what is technologically feasible. We will make three very specific assumptions to create an interpretable scenario, although it has to be stated that even this scenario is not technologically possible at the moment. First, sensors have a very large orthogonal chemical selectivity; each sensors reacts with a separate functional group but cannot deduce the length of the carbon backbone as this would require analysis of the mass. Second, sensors are unlimited in sensitivity. Third, we assume a limited number of VOCs: a 12x20 matrix, with carbon backbone length on the y-axis and functional chemical group on the x-axis, resulting in 188 VOCs (52 combinations between carbon backbone and functional group are chemically impossible). Figure 6 shows that the breathprint is expected to be more different in pneumonia than
in (early) ARDS especially if we keep the naturally occurring intra-person variation due to genetics and environmental exposure in mind [78].

**Figure 6:** Hypothetical eNose breathprint

The top figure shows the matrix of potential biological markers that were considered in this hypothetical example. The x-axis shows the different functional groups (= double binding, C carbon, N nitrogen, H hydrogen, S sulfide, ":1" gives the location of the functional group). The y-axis shows the carbon backbone (Arom = aromatic compound, for this example only one type). The lower left figure shows the breathprint that was identified for ARDS based on the GC-MS results in chapter 6. The lower right figure shows the breathprint of that was identified for bacterial pneumonia based on the literature study described in chapter 7. Even with a perfect chemically orthogonal sensor array based on these two dimensions the discrimination of pneumonia will outperform that of ARDS.
Multiple solutions are thinkable. For ARDS we could focus on other analytical technologies that allow for identification of single compounds or possibly increased understanding of olfactation in animals may provide new insights that can further enhance detection of VOCs by means of different sensor arrays. For example, most electronic nose technology relies on van der Waals forces and physical absorption [79]. These are the weakest and least selective of all intermolecular interactions. Novel concepts, such as optoelectronic noses based on chemoresponsive colorants utilize the chemical reactivity of the VOCs and thus allow for more sensitive and selective detection [79].

**Alternative technologies**

Several other technologies, besides GC-MS and eNose analysis, may be attractive for clinical validation studies in the near future. All technologies described here aim to detect a single compound. If a technology does not aim at that, I could include it in the section on electronic nose analysis as it is not relevant what type of “sensor input” is provided for pattern recognition. It should be noted that different advantages and limitations than those previously discussed could apply to those systems.

In GC-MS, chromatography separates the VOCs based on boiling point and chemical interaction with the stationary phase. If mass-spectrometry is to be used alone, separation has to be performed by another method. Additionally, electron ionization is traditionally used to generate ions. However, as described in **chapter 2**, this generates tens of fragments per VOC. Thus without chemical separation, this method of ionization would lead to chaos. Several methods have been used to overcome the problems of separation and ionization.

Proton transfer-reaction mass-spectrometry (PTR-MS) uses protons for chemical ionization [80]. This increases the mass of the VOC with one and adds a charge, which can be used for mass-spectrometric detection. Thus the molecule is not fragmented. Because multiple VOCs have the same nominal mass, they cannot be separated with this method. One solution is the use of time-of-flight mass-spectrometry (TOF-MS), which can more accurately separate masses [81]. As the elements do not have nominal masses exactly (e.g. oxygen has nominal mass 16 but the actual
mass is 15.9994) the accurate mass can be used to deduce the chemical formula of the molecule (but not the structural formula). One of the main limitations of PTR-MS is that not all molecules efficiently react with a proton, which limits the detection of some compounds. Furthermore, the PTR-MS machines that allow for accurate mass detection are very large and are not bedside devices.

Other ion-molecule reaction mass-spectrometry systems are also available and typically have other reactant molecules. For example, continuous breath analysis has been performed using krypton, mercury or xenon [82]. These systems have several advantages over PTR-MS with regard to the range of molecules that can be detected.

Selective ion-flow tube mass-spectrometry (SIFT-MS) also relies on chemical ionization but, in contrast to PTR-MS, uses multiple reactant molecules (H3O+, NO+ and O2+) [83, 84]. Furthermore, the addition of an ion-flow tube results in selective detection of molecules, because each molecule has an individual reaction rate coefficient and product ions. The use of Collision theory with reaction rate coefficients allows for absolute quantification of VOCs, which is a major advantage compared to any other analytical technique. SIFT-MS is a very versatile breath analyzer as many compounds can be detected specifically and quantitatively in real time. Furthermore, because the reactant molecules can be produced from water and air, a bottle of Helium gas is the only consumable that is required. Finally, the device is currently the size of a small table, is movable and can be used at the bedside. The cost per machine is currently the major drawback of this technology. With ion mobility spectroscopy (IMS) a sample of ionized VOCs is let into a drift chamber and driven towards a detector by an electric field [85, 86]. At the same time the ions are pushed towards the inlet by a drift gas (typically Helium). The time it takes an ion to travel through the drift tube, to the detector is a surrogate for the identity of the compound. Because the drift time is mostly non-specific for a VOC, detection of single molecular structures is not possible with traditional IMS. Several technological advances have increased the sensitivity and selectivity of the technique; a capillary column as separator [85, 87], a mass-spectrometer as detector [88] or changing field strengths for variable time periods [89] (High-field asymmetric-waveform ion-mobility spectrometry: FAIMS). Without a mass-spectrometer, this technique
can be miniturized and used as point of care machine. Therefore, IMS or similar technologies could be highly suitable for clinical practice if the problems with sensitivity and selectivity are overcome.

**Future applications**

The success of breath analysis as a clinical test for pulmonary injury or infection on the ICU is highly dependent on the availability, sensitivity, selectivity and ease of use analytical techniques. Several stages can be identified in the discovery, validation and valorization of any biological marker in general [68, 90]. In omic research, additional steps in the process of discovery are required as the markers that will be used in the diagnostic test are unknown at the beginning [91]. However, for breath research even these steps may be insufficient. Additional considerations should be given to the unknown biosynthesis of most volatile organic compounds and the difficulties with quantitative detection at the bedside. A 5 step program is proposed; from untargeted discovery to clinical test. Importantly, these steps are designed for analysis of specific VOCs only. For electronic nose approaches, without any considerations of the underlying changes in the VOC concentrations themselves, the traditional steps in assessment of diagnostic accuracy are sufficient [68, 90].

**Step 1: Discovery and model training**

The first step is identification of potential markers of the disease/syndrome. Most studies described in this thesis tried to take this step. I now recognize that several aspects are extremely important during discovery: (1) a (near) perfect reference standard for the clinical condition, (2) a clinically relevant control group, (3) semi-quantification of a wide range of VOCs in multiple platforms, (4) overlap in VOCs measured between the analytical platforms to minimize device errors and (5) a sufficient sample size. The clinical applicability of the analytical technique is of less importance in this step.

The combination of a good reference standard with a clinically relevant control group requires strong methodological efforts from clinicians. In the setting of the intensive care unit, comparing any ICU-patient to a healthy control does not contribute anything to discovery of clinically relevant biomarkers as any of thousands molecular pathways will be up- or down-
regulated during critical illness. This means that finding a significant difference does lead to the conclusion that this has anything to do with the disease of interest. The other way around, if a molecule is not found to be different between healthy individuals and patients with the disease this does not imply that it can never be used for clinical decision making within this specific population. This is in contrast to discovery studies where cases come from the general population (e.g. asthma or COPD). In that situation the likelihood of finding a true-positive or true-negative result when comparing cases to healthy controls are high compared to the above described ICU-situation. Thus even in this first phase, the included cohort should be a representation of a population that is clinically relevant (patients on the ICU) and the gold standard reference test should be applied to all included patients.

This step is also very challenging from an analytical chemistry perspective. The potential biomarkers are unknown and therefore an as wide range of molecules as possible should be measured. Furthermore, as described in this thesis, every analytical technique and platform has its selectivities and limitations. Therefore, preferably multiple methods and platform should be used on the same samples. In this thesis we always used one GC-MS platform under the assumption that this would be sufficient for discovery. However, I have to acknowledge that we could have missed potentially useful biomarkers by limiting our scope in that way. The usage of multiple GC-MS platforms would broaden the scope and limit the influence of measurement errors within a device. Addition of other analytical techniques such as SIFT-MS could also allow for the absolute quantification of some compounds and may be used to calibrate the other measurements.

In contrast to transcriptomics or genomics analysis that can reach low false-discovery rates with a small sample size because of relatively low heterogeneity, VOC analysis suffers from very high heterogeneity [92]. Furthermore, most VOCs in breath probably do not contain information on endo-genous processes as they come from the environment [92]. Thus, the sample size should be rather large compared to other “omics” approaches because of the high dimensionality of the predictor matrix, low signal-to-noise ratio and inter-person variation of exhaled VOCs.
Importantly, the research question in the first step is not: "can breath analysis accurately discriminate between patients with "X" and "Y"", but rather: "which VOCs in the breath are potential biomarkers to discriminate between "X" and "Y"" and "what algorithm can be used to discriminate between "X" and "Y" with these potential biomarkers?". The statistical considerations that should be taken with "omics" data in general and with breathomics specifically are recently described in excellent reviews and are outside the scope of this discussion [69, 91, 93]. Step 2a deals with assessment of the real diagnostic accuracy of a model, based on the biological markers that were discovered in step 1.

Figure 7: From untargeted discovery to clinical test

Step 2a: Validation of the diagnostic accuracy.

This is essentially an extension of the previous step and should be performed in a population that is selected with the same inclusion and exclusion criteria, using the same reference standard and the same
analytical techniques. However, in this step, biomarker discovery is no longer allowed. The only required analysis in this step is the blind application of the model that was developed in step 1. Therefore, this step could be published together with step 1 as one paper (see chapter 4 and chapter 6). In other words, the goal is to apply the classification algorithm on the discovered biomarkers to assess the real diagnostic accuracy within this population. It should be noted that reporting of diagnostic accuracy should be strictly following STARD guidelines [94].

**Step 2b: Biological translation**

A statistical association has been shown for several VOCs in step 1. However, as stated previously in this general discussion, the biological mechanism that leads to the formation of VOCs is important to estimate the post-test probability of a “real” discovery and to identify the population that is actually discriminated with this marker. A search in Pubmed, Pubchem, the Kyoto Encyclopedia of Genes and Genomes (KEGG) and other databases with information on biochemical and metabolic pathways can be used for identification of the biochemical pathways available to different cell types, which can be explored using a translational approach.

**Step 3: Technological translation**

VOCs were analyzed by sophisticated laboratory technologies that allow for identification of unknown compounds and can detect as many compounds as possible but are not available at the bedside. Furthermore, these technologies require specialized personal, storage and transport of breath samples and the analysis of the data is difficult. In this step, the technological translation from these laboratory techniques to a bedside test should be made. This is possible because the volatile biomarkers have been identified and analysis can be targeted rather than unbiased (as in step 1). Point of care (bedside) analysis removes the need to storage and transport of the sample and could thereby reduce the noise of the signal. Several technologies might be used as a rapid, bedside test. The most applicable technology mostly depends on the VOCs that were found in step 1. Several candidate techniques are: PTR-MS, SIFT-MS, IMR-MS, spectroscopy and semi-selective sensor technology.
**Step 4: Clinical validation of the point of care device**

In this step the diagnostic accuracy of the breath test is validated in a large, non-selective cohort. The technology adapted or developed in step 4 is used for detection of the VOCs. The primary outcome of this study is the discrimination that can be obtained by the breath test. This is the last opportunity to re-calibrate the breath test so that the post-test probabilities reflect the observed probabilities. Using this re-calibrated algorithm, test characteristics can be obtained at several cut-off values. If the measures of diagnostic accuracy are sufficient, they can be implemented in clinical decision support. In other words, what action is recommended if the test scores are above a certain level (in combination with several clinical parameters)?

**Step 5: Implications for patient care**

The implications for patient care are the most important evaluation of the added value of a diagnostic test. For example, a test that can discriminate between patients with and without positive BAL-cultures within a population that is clinically suspected of VAP could reduce the use of antibiotic therapy. Another clinical dilemma is the early recognition of lung injury and the start of tailored therapy as described previously in the general discussion of this thesis. It could be argued that diagnosis alone is a sufficient endpoint for a diagnostic test. However, providing a name for a condition is not the goal of medicine; we aim to improve outcomes. Therefore, the implications for patient care should not be assumed from the diagnostic accuracy of the test but should be empirically evaluated in a randomized clinical trial [95].

**Summary**

In this thesis we described the analysis of exhaled breath for the diagnosis of ARDS and pneumonia in ventilated intensive care unit patients. A novel, simplified sample methodology was developed that allows for collection of breath in large populations of ventilated patients (chapter 3). We relied on gas-chromatography and mass-spectrometry for the discovery of volatile biomarkers. In an animal model of acute pulmonary inflammation animals that did or did not receive lipopolysaccharide could be discriminated within hours (chapter 4). We also identified octane, 3-methyl-heptane and
acetaldehyde as markers of ARDS (chapter 6). We confirmed that ARDS could be discriminated from controls by means of electronic nose analysis of the breath, although the diagnostic accuracy was less than with GC-MS analysis (chapter 5). A review of the literature showed that multiple VOCs may be used to identify the presence of pathogens (chapter 6). Although we observed that pneumonia is associated with a changed VOC-profile of tracheal aspirates (chapter 8) and with a decreased concentration of 1-propanol in breath (chapter 9), the specific findings from chapter 6 could not be confirmed.

In this chapter, a biological interpretation of the results of the previous chapters was given. Furthermore, a roadmap for future studies on breath analysis for the diagnosis of ARDS and pneumonia in ventilated patients was sketched.

References


Chapter 11.

Nederlandse samenvatting

Lieuwe DJ Bos
Inleiding

Ernstig zieke patiënten worden op de intensive care (IC) opgenomen om zorg te ontvangen die op andere afdelingen niet leverbaar is en om uitgevallen orgaanfuncties te ondersteunen. IC-patiënten kunnen mechanisch worden beademend als zij respiratoir insufficiënt worden, bijvoorbeeld omdat zij acute longschade (acute respiratory distress syndrome; ARDS) of een longinfectie (pneumonie) hebben. Daarnaast kunnen zowel ARDS als pneumonie zich ontwikkelen gedurende de periode dat iemand mechanisch wordt beademd. ARDS en pneumonie zijn beide geassocieerd met een ontstekingsreactie. In dierstudies kan deze reactie worden waargenomen in het systemische (bloed) en pulmonale compartiment. In monsters van patiënten ziet men echter dat de lokale markers voor ontsteking beter toestaan om te discrimineren tussen patiënten met en zonder longschade of infectie. Een belangrijk element hierbij blijkt de keuze voor de controle groep (de groep zonder de ziekte) te zijn. Lokale markers van inflammatie discrimineren steeds beter naarmate de controle groep klinisch relevanter wordt. Dus wanneer men geïnteresseerd is in biologische markers voor ARDS of pneumonie kan men zich het beste focussen op monsters vanuit het pulmonale compartiment. De meest betrouwbare manier om dat momenteel te doen is bronchoalveolaire lavage. Bij deze handeling spoelt men de lagere luchtwegen met een zout-oplossing om de vloeistoflaag die zich binnen in de long bevindt los te spoelen. Hierin kunnen vervolgens markers van ontsteking worden bepaald. Deze verrichting is echter niet zonder risico’s voor de patiënt en kan zeker niet vaak worden herhaald. Daarom was het centrale postulaat dat aan de basis stond van dit proefschrift: er is behoefte aan een niet-invasieve methode om pulmonale moleculaire markers te meten waarmee ARDS en pneumonie kunnen worden voorspeld en gediagnosticeerd in beademende intensive care patiënten.

Naast enkele veel voorkomende moleculen zoals stikstof, zuurstof en koolstofdioxide bevat adem ook vluchtige organische componenten (VOCs) in heel lage concentraties (één molecuul per miljoen of biljoen). Deze moleculen representeren (fragmenten van) metabole producten. Ze kunnen van systemische origine zijn en via de bloedbaan naar de long zijn getransporteerd of lokaal in de long worden geproduceerd. Verscheidene mechanismen kunnen leiden tot de formatie van VOCs.
Ketonen worden gemaakt in het centrale metabolisme, als onderdeel van de energievoorziening. Oxidatie van vetzuren kan resulteren in alkanen, alkenen en aldehydes. In vitro experimenten suggereren dat witte bloedcellen VOCs kunnen produceren. VOCs die stikstof en sulfide atomen bevatten worden normaal gesproken uit het lichaam verwijderd door de lever en de nier. De concentratie van deze stoffen in de adem kan stijgen wanneer de functie van deze organen is verstoord. Micro-organismen kunnen ook bijdrage aan de VOCs die in de uitademingslucht gevonden worden. Bacteriën in de darmen produceren vluchtige moleculen gedurende fermentatie. Relevanter voor pneumonie is dat bacteriën in de long ook VOCs kunnen produceren, al dan niet in interactie met de gastheer. Tot slot worden vele componenten uit de omgeving opgenomen; ze worden ingeademd, komen uit voedsel of zijn een reflectie van medicatie.

Vluchtige organische componenten kunnen op vele wijze worden gedetecteerd. In dit proefschrift hebben wij ons op twee methodes gefocust: a) gaschromatografie en massaspectrometrie (GC-MS) voor de scheiding, identificatie en kwantificatie van potentiële vluchtige biologische markers en b) elektronische neus analyse voor de herkenning van complexe mengsels van VOCs door middel van het vatten van de “moleculaire vingerafdruk”. Deze afdruk representeert een patroon voor ARDS of pneumonie die gebruikt kan worden voor empirische classificatie. Om uitademingsluchtanalyse toe te staan in ernstig zieke, beademde intensive care patiënten moest een nieuw, versimpeld adem collectie systeem worden ontwikkeld. GC-MS werd gebruikt om vluchtige componenten te identificeren die wellicht nuttig kunnen zijn in de vroege herkenning en diagnose van ARDS en pneumonie. We hebben ook de accuraatheid van een commercieel beschikbare elektronische neus voor de empirische diagnose van ARDS en pneumonie onderzocht. De resultaten en conclusies van dit onderzoek zullen op de volgende pagina’s worden samengevat. De samenvatting is apart uiteengezet voor ARDS en pneumonie, zoals dit ook in de rest van dit proefschrift is gebeurd.

**Acute longschade**

Een nieuwe, versimpelde methode voor de collectie van adem was ontwikkeld. Deze methode stond toe dat adem snel en veilig verzameld kan worden in mechanisch beademde intensive care patiënten (hoofdstuk
Een zijdelings lekstroompje van lucht zorgt ervoor dat de VOCs kunnen neerslaan op een absorberend materiaal waarmee GC-MS analyses uitgevoerd kunnen worden. Directe analyse aan het bed kan ook worden uitgevoerd met behulp van een elektronische neus (hoofdstuk 4). Deze methode gaf reproduceerbare resultaten. Meerdere VOCs die in hoge concentraties door de ventilator en slangensysteem werden afgegeven werden geïdentificeerd. Deze VOCs konden a priori worden geëxcludeerd als potentiele markers. In twee diermodellen (hoofdstuk 5) waarin we ratten intraveneus of intratracheaal lipopolysaccharide (LPS) hebben toegediend om het verloop van een ontstekingsreactie na te bootsen vonden we dat VOCs concentraties in de adem veranderden gedurende de ontwikkeling van pulmonale ontsteking en schade. Meerdere stoffen overlapten tussen de twee diermodellen voor longschade en kunnen wellicht worden gezien als kandidaat markers voor een ontstekingsreactie. Interessant genoeg, hexanal, pentadecaan, en 6,10-dimethyl-5,9-undecadien-2-one werden in lagere concentraties gevonden na LPS toediening. Dit hadden wij niet geanticipeerd omdat deze stoffen meestal worden geassocieerd met oxidatieve stress, wat tot een blijven zou moeten leiden na LPS toediening. In hoofdstuk 4 beschreven we dat ademanalyse met een commercieel verkrijgbare elektronische neus kan worden gebruikt om te discrimineren tussen intensive care patiënten met en zonder ARDS met redelijke accuratesse. De diagnostische accuratesse nam toe met de ernst van ziekte. Desalniettemin, moesten wij concluderen dat de momenteel gebruikte elektronische neus technologie niet toepasbaar in de klinische situatie omdat de test accuratesse te laag was en omdat de sensoren een duidelijke drift over tijd lieten zien. In hoofdstuk 6 gebruikten we een zelfde studie opzet als in hoofdstuk 4 maar pasten GC-MS analyse toe in plaats van elektronische neus analyse. We vonden dat het gecombineerde signaal van drie metabolieten (octaan, 3-methyl heptaan en acetaldehyde) in de adem kon worden gebruikt om patiënten met en zonder ARDS te discrimineren met goede accuratesse. Deze accuraatheid bleef behouden in een temporaal extern validatie cohort. Dit limiteert de kans dat onze bevindingen op toeval berustten. Bovendien bleek het signaal in de uitademingslucht de diagnostische accuratesse van een bestaande klinische voorspellingse score te verbeteren.

Tezamen laten deze data zien dat specifieke VOCs in de uitademingslucht
gebruikt kunnen worden om patiënten met ARDS te discrimineren van patiënten zonder ARDS. Dit suggereert dat ademanalyse gebruikt kan worden als diagnostische test voor ARDS.

**Pneumonie**


De belangrijkste limitatie van alle geïncludeerde studies was dat de evaluatie van de VOCs *in vitro* werd gedaan zonder dat de bacteriën een groeimedium kregen dat enigszins leek op de condities die zij *in vivo* zullen tegenkomen. Daarom evalueerden wij de diagnostische accuratesse van VOC analyse voor de diagnose van VAP zonder gebruik te maken van een artificieel groeimedium in **hoofdstuk 8**. In deze studie werden tracheaal aspiraten verzameld om de drie dagen in mechanisch beademde patiënten zonder tekenen van pneumonie. Sommige van deze patiënten ontwikkelden vervolgens een pneumonie; zij werden gebruikt als zieke populatie. Tracheaal aspiraten werden ontdooid en geanalyseerd door middel van elektronische neus en GC-MS. De elektronische neus analyse liet zien dat patiënten met pneumonie konden worden onderscheiden van patiënten zonder pneumonie en dat dit signaal al toenam voor het moment van diagnose. Patiënten met gekoloniseerde luchtwegen maar zonder infectie werden geclassificeerd als hebbende geen pneumonie in
deze studie. Dit suggereert dat de *in vivo* markers voor pneumonie niet zijn gerelateerd aan bacteriële aanwezigheid en groei *per se*. Dat zou kunnen betekenen dat de moleculaire vingerafdruk die werd gedetecteerd door de elektronische neus vooral toegedragen zou moeten worden aan de reactie van de gastheer of dat bacteriën andere VOCs produceren gedurende invasieve groei (in tegenstelling tot kolonisatie). In die studie hebben wij ook getracht de VOCs te identificeren die in hogere of lagere concentraties aanwezig zijn tijdens pneumonie. Echter, de GC-MS analyses waren zeer vaak onsuccesvol doordat er water op het opslagmateriaal was geabsorbeerd en doordat er een extreem wijde spreiding in de concentraties zat tussen de verschillende samples. Hierdoor overlaadden sommige samples de massaspectrometer terwijl andere alleen zeer lage intensiteit pieken lieten zien. In *hoofdstuk 9* hebben we de associatie tussen VOCs, pneumonie en bacteriële aanwezigheid verder geëvalueerd, alleen nu *in vivo*. Voor deze studie werd de ademverzameling en analyse uitgevoerd volgens de methodologie die in *hoofdstuk 3* werd beschreven. Patiënten werden verdeeld in degenen die zeker een pneumonie hadden bij opname op de intensive care en in degenen zonder enige verschijnselen daarvan. 1-Propanol was significant lager in de adem van patiënten met een pneumonie dan in hen die geen pneumonie hadden. Patiënten met en zonder positieve kweek van tracheaal secreet werden ook vergeleken. 1-Pentanol en heptanal waren significant lager in patiënten met positieve kweken. Tot slot werden de VOCs in adem gecorreleerd aan markers van ontsteking in het bloed. Geen van VOCs liet een significante correlatie zien met interleukine 1 beta, interleukine 6, tumor necrosis factor alpha en granulocyte-macrophage colony-stimulating factor. We observeerden wel correlaties tussen verschillende VOCs en interleukine 8, 10 en 13 en interferon gamma. De positieve correlatie met interferon gamma was de meest uitgesproken.

Tezamen demonstreren deze data dat specifieke VOCs worden geproduceerd door bacteriële stammen, maar dat deze resultaten niet gemakkelijk te vertalen zijn naar een klinische test voor pneumonie.
Part V

Appendices
Appendix A.

Acknowledgements
Acknowledgements

If you rushed right to the acknowledgements, skipping all scientific chapters, like many of us do, I have to disappoint you.

This thesis is the result of effort from many, many people and even my most thorough effort at listing everyone would not result in a complete overview of whom contributed.

Therefore, plain and simple:

Anyone who participated in the studies described in this thesis

   As a researcher
   As a physician
   As a patient

Anyone who argued with me

Anyone who provided moral support

Thank you.
Personal Acknowledgement
Appendix B.

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http://www.ncbi.nlm.nih.gov/pubmed/?term=Bos%2C+Lieuwe%5BAuthor%5D

or scan the following 2D barcode with your smartphone
Appendix C.

Curriculum Vitae
Curriculum Vitae

Lieuwe Durk Jacobus Bos werd geboren op 16 Augustus 1988 te Alkmaar. Hij behaalde zijn Atheneum diploma in 2006 op Openbare Scholen Gemeenschap Willem Blaeu. In datzelfde jaar begon hij met de opleiding Geneeskunde aan de Universiteit van Amsterdam nadat hij hiervoor door middel van een decentrale selectie ronde was geselecteerd. In het derde jaar begon hij met een parttime onderzoekstage bij de afdeling Longziekten, onder supervisie van Professor Peter Sterk. Dit onderzoek heeft hij uitgebreid tot een wetenschappelijke stage bij de afdeling Longziekten en de afdeling Intensive Care onder supervisie van Professor Marcus Schultz en Professor Peter Sterk. In 2010 was Lieuwe één van de winnaars van de AMC PhD Scholarship waarmee hij een promotietraject van vier tegemoet ging. Diezelfde zomer behaalde hij zijn doctoraal examen Geneeskunde. Gedurende zijn promotietraject op de afdelingen Intensive Care en Longziekten van 2010-2014 heeft hij het onderzoek uitgevoerd dat in dit proefschrift staat beschreven. Tijdens deze periode heeft hij daarnaast onderzoek uitgevoerd bij de afdeling Analytische Chemie in het Science Park, onder supervisie van Professor Hans-Gerd Janssen. Bovendien heeft hij een samenwerkingsverband opgezet met Hospital Parc Tauli, nabij Barcelona in Spanje. Dat onderzoeksproject resulteerde in de toekenning van de “Young Investigator Award” door de European Society for Intensive Care Medicine aan Lieuwe. In het kader van deze samenwerking hebben meerdere uitwisselingen tussen Spanje en Nederland plaats gevonden. Vanaf 1 mei 2014 is Lieuwe parttime werkzaam als Post-Doc, superviseert hij een promovendus en leidt hij meerdere internationale studies. In de nu overvloedige vrije tijd zal hij zich richten op andere passies: sporten (triathlon) en reizen. In Mei 2014 plaatste hij zich voor de Ironman 70.3 World Championship (Mont Tremblant, Canada). In 2015 hoopt hij zijn co-schappen te beginnen om alsnog zijn artsentitel te behalen.
Curriculum Vitae

Lieuwe Durk Jacobus Bos was born on the 16th of August 1988 in Alkmaar, the Netherlands. He received his “Ateneum” diploma in 2006 at the “Openbare Scholen Gemeenschap Willem Blaeu”. In the same year he began to study Medicine at the University of Amsterdam. In the third year he started with a part-time research project at the respiratory medicine department, under supervision of Professor Peter Sterk. That line of research evolved into a research internship at the department of respiratory medicine and at the intensive care unit, under supervision of both Professor Peter Sterk and Professor Marcus Schultz. Lieuwe was one of the recipients of the “AMC PhD Scholarship” in 2010, which allowed him to do a four year PhD project. That summer he also completed his “doctoraal” exam in Medicine.

Lieuwe performed the research that is described in this thesis during his PhD project on the departments of intensive care and respiratory medicine from 2010-2014. Besides, he also performed studies in the department of analytical chemistry, under supervision of Professor Hans-Gerd Janssen. Furthermore, he established a research collaboration with Hospital Parc Tauli, close to Barcelona in Spain. Lieuwe was awarded the “Young Investigator Award” from the European Society for Intensive Care Medicine for that collaboration in 2012. That project led to fruitful exchanges between Spain and the Netherlands.

From May 2014, Lieuwe works part-time as a Post-Doc researcher, supervises a PhD-student and oversees multiple International studies. In the now plentiful spare time he focusses on other passions: sports (triathlon) and traveling. In May 2014 he qualified for the Ironman 70.3 World Championship (Mont Tremblant, Canada). He hopes to start his clinical internships in 2015 and at last pursue a medical degree.
Appendix D.

PhD Portofolio
Name PhD student: Lieuwe DJ Bos
PhD period: 1st May 2010 to 1st May 2014
Name PhD supervisor: Marcus J Schultz and Peter J Sterk

1. PhD training

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- American Thoracic Society International Conference
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- American Thoracic Society International Conference
  “Continuous monitoring of Pulmonary Inflammation and Injury by Electronic Nose analysis” (Poster discussion)
- Breath Summit
  “Exhaled breath profiling for Diagnosing ARDS in Intubated and Mechanically Ventilated Patients” (Poster presentation)
- European Society of Intensive Care Medicine International Conference
  “Volatile Metabolite Fingerprints of Tracheal Aspirates Discriminates patients with VAP from Controls” (Oral Presentation)
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<td>- Respiratory Research Meeting (weekly)</td>
<td>2010-2014</td>
<td>18</td>
</tr>
<tr>
<td>- Intensive Care Research Meeting (weekly)</td>
<td>2010-2014</td>
<td>18</td>
</tr>
<tr>
<td>- Laboratory of Experimental Intensive Care and Anaesthesia Meeting (weekly)</td>
<td>2010-2014</td>
<td>18</td>
</tr>
</tbody>
</table>
2. Teaching

Student coaching and mentoring
- Bachelor thesis (Medicine) 2010 1.0
- Bachelor thesis (Medicine) 2010 1.0
- Research Internship (Medicine) 2011 1.0
- Research Internship (Medicine) 2012 1.0
- Research Internship (Medicine) 2012 2.0
- Bachelor Thesis (Medical Informatics) 2012 1.0
- Research Internship (Medicine) 2013 1.0
- Master Thesis (Medical Informatics) 2013 2.0
- Master Thesis (Medicine) 2014 1.0
- Master Thesis (Medical Informatics) 2014 1.0

3. Parameters of Esteem

Grants
- AMC PhD Scholarship 2010
- Four year unrestricted research grant from Philips Medical Research 2010
- Institut-Merieux research grant 2012
- Marie Curie (FP7-PEOPLE-2013-IAPP) grant 2013

Awards and Prizes
- American Thoracic Society International Trainee Travel Award 2010
- ATS Public Advisory Roundtable Travel Award from the ARDS foundation 2011
- ATS Critical Care Assembly Travel Award from the ARDS foundation 2012
- European Society of Intensive Care Medicine Young Investigator Award 2012
- ATS Abstract award from Critical Care Assembly 2013
- Poster award during Breath Summit 2013